



**Harper Adams  
University**

A Thesis Submitted for the Degree of Doctor of Philosophy at  
Harper Adams University

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HARPER ADAMS UNIVERSITY

**“*Catch me if you can*” – improving  
monitoring and control of vine weevil  
(*Otiorhynchus sulcatus*) in soft fruit and  
ornamental crops**

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A thesis submitted in partial fulfilment of the requirements for the  
award of the degree of Doctor of Philosophy

28<sup>th</sup> September 2024

Harper Adams University, Edgmond, Shropshire, United Kingdom

UTILE + DULCI

## **Declaration**

I hereby declare that this thesis is my original work and represents the research and efforts carried out solely by me, or predominantly by myself in collaboration with others, as duly acknowledged. I further confirm that no part of this work has been submitted for any other degree or qualification.

Eugenia Fezza

September, 2024

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## Thesis Abstract

Vine weevil (*Otiorhynchus sulcatus* F.; Coleoptera: Curculionidae) is widely considered to be one of the most important soft-fruit and ornamental crop pests worldwide. Management options for vine weevil populations have historically relied on conventional synthetic insecticides. Much progress has been made in developing alternatives to conventional synthetic insecticides for vine weevil control. Research has focused on the use of entomopathogenic nematodes (EPNs) so that standard practice is now to use EPNs, such as *Steinernema kraussei* or *Heterorhabditis bacteriophora*, to control vine weevil larvae. However, despite progress in developing sustainable control methods, less progress has been made in developing the other components required to form a holistic integrated pest management (IPM) programme for this pest.

The series of experiments presented in this thesis seek, through the development of an improved monitoring tool as well as the application of a biopesticide as an alternative to the use of synthetic insecticides, to offer growers effective tools with which to monitor and control vine weevil. In particular, the influence of visual cues on vine weevil behaviour is investigated to develop an improved monitoring tool of this pest. Similarly, the chemical ecology of this species was studied to identify semiochemicals that can be used as a lure to improve monitoring tool efficacy. To achieve this, host and non-host plants as well as vine weevil produced volatiles were investigated. In addition, a bioinsecticide based on garlic extracts (Pitcher GR<sup>®</sup>) was evaluated under laboratory and glasshouse conditions in order to determine its potential as a vine weevil control. Finally, the efficacy of EPNs at controlling vine weevil larvae is assessed in six commercially available peat-free growing media.

Results showed that visual (monitoring tool shade/colour, height and diameter as well as the effect of monitoring tool density) and olfactory (host plant and apple sauce) cues influence vine weevil monitoring tool efficacy. The garlic-based product was effective against vine weevil eggs and larvae under laboratory conditions, but its efficacy under glasshouse conditions requires further investigation to optimise its use against vine weevil. When the efficacy of EPNs was evaluated in six commercially available peat alternative growing media, EPNS were found to survive in all the growing media types. However, dispersal was affected by growing media, with Coir and John Innes Number 2 having a negative effect on EPNs. In addition, when *S. kraussei* was tested under glasshouse conditions, the EPN was effective at controlling vine weevil in all growing media tested.

This study provides the basis to develop an enhanced monitoring tool that incorporates both visual and olfactory cues, while also promoting the effective use of biopesticides (e.g., garlic-based bioinsecticides) and biological controls (EPNs) for vine weevil control. However, further research is necessary to optimise vine weevil monitoring and management strategies.

## Statement of Publications

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## Tables of Contents

<b>Declaration</b> .....	<b>i</b>
<b>Acknowledgements</b> .....	<b>ii</b>
<b>Thesis Abstract</b> .....	<b>iv</b>
<b>Statement of Publications</b> .....	<b>v</b>
<b>Tables of Contents</b> .....	<b>vi</b>
<b>List of Figures</b> .....	<b>xii</b>
<b>List of Tables</b> .....	<b>xviii</b>
<b>Chapter 1: General Introduction</b> .....	<b>1</b>
1. Introduction: Thesis Overview.....	1
2. A Review of the Biology, Ecology and Control of Vine Weevil.....	4
2.1 Distribution .....	5
2.2 Life Cycle .....	5
2.2.1 Eggs .....	6
2.2.2 Larvae .....	6
2.2.3 Pupae .....	7
2.2.4 Adults .....	7
2.3 Host Range .....	8
2.4 Economic Importance .....	9
2.5 Endosymbionts .....	10
2.6 Integrated Vine Weevil Management .....	10
2.6.1 Monitoring.....	11
2.6.1.1 Detection of Larvae.....	11
2.6.1.2 Directly Counting of Adults .....	12
2.6.1.3 Indicator Plants .....	12
2.6.1.4 Monitoring Tools.....	12
2.6.1.5 Semiochemical Lures.....	13
2.6.2 Forecasting.....	14
2.6.3 Thresholds.....	15
2.6.4 Prevention .....	15
2.6.5 Control.....	16
2.6.5.1 Synthetic Chemical Insecticides.....	16
2.6.5.2 Bioprotectants.....	17
2.6.5.2.1 Microbial Bioprotectants .....	17
2.6.5.2.2 Natural Substance Bioprotectants .....	19

2.6.5.3 Invertebrate Biological Control Agents .....	20
3. Thesis Aims and Objectives .....	23
<b>Chapter 2: Effect of Visual Cues on Monitoring Tool Efficacy.....</b>	<b>24</b>
Abstract .....	24
1. Introduction.....	25
2. Material and Methods .....	26
2.1 Insects .....	26
2.2 Laboratory Experiments .....	27
2.2.1 Monitoring Tool Colour (Light vs Dark).....	27
2.2.2 Monitoring Tool Colour (Multiple) .....	28
2.2.3 Monitoring Tool Height .....	30
2.2.4 Monitoring Tool Entrance Position.....	30
2.3 Glasshouse Experiments .....	31
2.3.1 Experimental Set-Up.....	31
2.3.2 Monitoring Tool Colour (Light vs Dark).....	32
2.3.3 Monitoring Tool Height .....	33
2.3.4 Monitoring Tool Diameter .....	33
2.3.5 Monitoring Tool Density.....	34
2.3.6 Effect of Plant Density on Vine Weevil Monitoring Tool Performance .....	35
3. Statistical Analysis.....	35
4. Results .....	36
4.1 Laboratory Experiments .....	36
4.1.1 Monitoring Tool Colour (Dark vs Light).....	36
4.1.2 Monitoring Tool Colour (Multiple) .....	37
4.1.3 Monitoring Tool Height .....	38
4.1.4 Monitoring Tool Entrance Positions .....	38
4.2 Glasshouse experiments.....	39
4.2.1 Monitoring Tool Colour (Dark vs. Light).....	39
4.2.2 Monitoring Tool Height .....	39
4.2.3 Monitoring Tool Diameter .....	40
4.2.4 Monitoring Tool Density.....	40
4.2.5 Effect of Plant Density on Vine Weevil Monitoring Tool Performance .....	41
5 Discussion.....	41
<b>Chapter 3: Effect of Photoperiod and Olfactory Cues on Vine Weevil Behaviour and Monitoring Tool Efficacy .....</b>	<b>46</b>
Abstract.....	46

1. Introduction.....	46
2. Materials and Methods .....	49
2.1 Insects .....	49
2.2 Laboratory Experiments .....	49
2.2.1 Y-tube Olfactometer Bioassay.....	49
2.2.2 Effect of Light.....	50
2.2.3 Effect of Light and Photoperiod.....	51
2.3 Glasshouse Experiments .....	51
2.3.1 Experimental Set-up .....	51
2.3.2 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance.....	52
3. Statistical Analyses.....	53
4. Results .....	53
4.1 Laboratory Experiments .....	53
4.1.1 Effect of Light.....	53
4.1.1.1 'Night' .....	53
4.1.1.2 'Day Light' .....	53
4.1.1.3 'Dawn Light' .....	55
4.1.2 Effect of Light and Photoperiod.....	55
4.1.2.1 Standard Photoperiod .....	55
4.1.2.2 Reversed Period .....	56
4.2 Glasshouse Experiments .....	57
4.2.1 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance.....	57
5. Discussion.....	58
<b>Chapter 4: Effect of Apple Volatiles on Vine Weevil Adults Behaviour .....</b>	<b>62</b>
Abstract.....	62
1. Introduction.....	62
2. Materials and Methods .....	64
2.1 Insects .....	64
2.2 Laboratory Experiments .....	65
2.2.1 Collection of Apple Sauce Volatiles.....	65
2.2.2 Analysis by GC-MS.....	65
2.2.3 Analysis by GC-EAD.....	65
2.2.4 Y-tube Olfactometer Bioassay.....	66
2.3 Glasshouse Experiments .....	67
2.3.1 Experimental Set-up .....	67
2.3.2 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance.....	67

3. Statistical Analyses.....	69
4. Results .....	69
4.1 Laboratory Experiments .....	69
4.1.1 GC/EAG and GC/MS Analyses of Apple Sauce Volatiles .....	69
4.1.2 Vine Weevil Dose-Responses to Apple Sauce.....	71
4.1.3 Vine Weevil Response to Olfactory Cues.....	71
4.2 Glasshouse Experiments .....	72
5. Discussion.....	74
<b>Chapter 5: Investigating the Effect of a Garlic-Based Product, Pitcher GR<sup>®</sup>, on Vine Weevil and Its Compatibility with Entomopathogenic Nematodes.....</b>	<b>78</b>
Abstract.....	78
1. Introduction.....	79
2. Materials and Methods .....	81
2.1 Insect Cultures .....	81
2.2 Begonia and Primula plants .....	81
2.3 Entomopathogenic nematodes solution.....	81
2.4 Bioinsecticide Treatment.....	82
2.5 Toxicity Screening on Vine Weevil Eggs .....	82
2.5.1 Egg Collection .....	82
2.5.2 Bioassay Experimental Units .....	83
2.5.3 Contact Toxicity Bioassay .....	84
2.5.4 Fumigant Toxicity Bioassay.....	84
2.5.5 Combined Contact and Fumigant Toxicity Bioassay.....	84
2.5.6 Dose Response Toxicity Bioassay .....	85
2.6 Toxicity Screening on Vine Weevil Larvae .....	85
2.6.1 Larva production.....	85
2.6.2 Bioassay Experimental Units .....	85
2.6.3 Contact Toxicity Bioassay .....	86
2.6.4 Fumigant Toxicity Bioassay.....	86
2.6.5 Combined Contact and Fumigant Toxicity Bioassay.....	87
2.7 Growing Media Bioassay.....	87
2.8 Compatibility Bioassay .....	87
2.9 Glasshouse Experiments .....	88
2.9.1 Combined Contact and Fumigant Toxicity Bioassay.....	88
2.9.2 Efficacy Bioassay.....	89
3. Statistical Analysis.....	91
4. Results .....	91

4.1 Vine Weevil Eggs .....	91
4.1.1 Contact Toxicity Bioassay .....	91
4.1.2 Fumigant Toxicity Bioassay.....	91
4.1.3 Combined Contact and Fumigant Toxicity Bioassay.....	92
4.1.4 Dose Response Toxicity Bioassay .....	92
4.2 Vine Weevil Larvae .....	93
4.2.1 Contact Toxicity Bioassay .....	93
4.2.2 Fumigant Toxicity Bioassay.....	93
4.2.3 Combined Contact and Fumigant Toxicity Bioassay.....	94
4.3 Combined Contact and Fumigant Bioassay with Growing Media.....	94
4.4 Compatibility Bioassay .....	95
4.5 Glasshouse Experiments .....	96
4.5.1 Combined Contact and Fumigant Bioassay .....	96
4.5.2 Efficacy Bioassay.....	96
5. Discussion.....	98
<b>Chapter 6: Efficacy of Entomopathogenic Nematodes in Peat-Free Growing Media. 103</b>	
Abstract .....	103
1. Introduction.....	104
2. Materials and Methods .....	105
2.1 Insect Cultures .....	105
2.2 EPN Dilution.....	106
2.3 Growing Media.....	106
2.4 Laboratory Experiments .....	108
2.4.1 Survival Bioassay .....	108
2.4.2 Dispersal Bioassay .....	108
2.5 Glasshouse experiments.....	109
2.5.1 Begonia Plants .....	109
2.5.2 Efficacy Bioassay.....	110
3. Statistical Analysis.....	112
4. Results .....	112
4.1 Laboratory Experiments .....	112
4.1.1 Survival Bioassay .....	112
4.1.2 Dispersal Bioassay .....	114
4.1.2.1 Parasitism Bioassay.....	115
4.2 Glasshouse Experiments .....	115
4.2.1 Efficacy Bioassay.....	115

4.2.2 Larval Weight.....	117
5. Discussion.....	117
<b>Chapter 7: General Discussion .....</b>	<b>122</b>
1. Current Knowledge and Future Prospectives.....	122
1.1 The Importance of Visual Characteristic of Vine Weevil Monitoring Tool Efficacy..	122
1.2 Potential of Semiochemicals from Host Plants and Conspecifics That May Be Used to Improve Monitoring Tool Efficacy .....	123
1.3 Investigate the Potential of Semiochemicals from Apple-Based Products That May Be Used to Improve Monitoring Tool Efficacy.....	125
1.4 Efficacy of a Commercially Available Garlic-Based Bioinsecticide Against Vine Weevil Eggs and Larvae .....	126
1.5 Efficacy of Entomopathogenic Nematodes Against Vine Weevil Larvae When Applied to Peat-Free Growing Media .....	127
2. Developing Improved Tools with Which to Reduce Vine Weevil Damage .....	128
3. Integrated Available Management Tools into IPM Programmes.....	130
4. Conclusion.....	132
<b>References .....</b>	<b>134</b>

## List of Figures

<b>1- 1.</b> Typical life cycle of vine weevil under UK conditions (Source: Penny Greeves).....	5
<b>1- 2. a)</b> Eggs (Source: Bennison <i>et al.</i> , 2018), <b>(b)</b> larva (Source: author's own) and <b>(c)</b> pupa (Source: author's own) of vine weevil.....	6
<b>1- 3. a)</b> Adult vine weevil; <b>b)</b> Typical intraspecific aggregation between vine weevil adults observed in laboratory condition (Source: author's own and Dr Joe Roberts, respectively)....	8
<b>2- 1.</b> Schematic diagram showing the arrangement within each mesh cage for the monitoring tool colour (black vs. white) experiment. Monitoring tool positions are shown in circles and the vine weevil release point using an x mark.....	27
<b>2- 2.</b> Arrangement within each mesh cage for the monitoring tool colour (black vs. white) experiment with different colour backgrounds: white (a) or black (b).....	28
<b>2- 3.</b> Arrangement within each mesh cage for the monitoring tool colour (multiple) experiment.....	29
<b>2-4.</b> Reflectance values from spectral analysis of coloured paper cups used in trapping experiments and measured using Flame Miniature Spectrometer, Ocean Insight, Duiven, The Netherlands.....	29
<b>2- 5.</b> Monitoring tools with four different entrance configurations used to test the effect of monitoring tool entrance position on vine weevil adults: (a) four 1 cm <sup>2</sup> entrances equally distanced from one another around the monitoring tool base, b) one 1 cm high continuous entrance around the monitoring tool base, (c) four 1 cm <sup>2</sup> entrances equally distanced from one another 8.3 cm from the monitoring tool base and (d) four 1 cm <sup>2</sup> entrances equally distanced from one another at the top of the monitoring tool.....	31
<b>2- 6.</b> Schematic diagram showing the arrangement within each tent cage for the monitoring tool colour/ shade experiment. The vine weevil release point is shown by x.....	32
<b>2- 7.</b> Schematic diagram showing the arrangement within each tent cage for the monitoring tool (A, B, C) and plant (D, E, F) density experiment. The vine weevil release point is shown by x.....	34
<b>2- 8.</b> Percentage of vine weevil adults recorded in monitoring tools painted black or white when released individually (number of replicate days = 10) into a cage with a black background or white background (Binomial exact test: $p < 0.05$ ).....	36

<b>2- 9.</b> Percentage of vine weevil adults recorded in monitoring tools painted black or white when released as a group of five individuals (number of replicate days = 5) into a cage with a black background or white background (Binomial exact test: $p < 0.05$ ).....	37
<b>2- 10.</b> Percentage of vine weevil adults recorded in monitoring tools painted blue, green, red, yellow, black or white when released (A) individually (number of replicate days = 10) or (B) as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test: $p < 0.05$ ).....	37
<b>2- 11.</b> Percentage of vine weevil adults recorded in monitoring tools with a height of 3, 6 or 11.30 cm when released as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test: $p < 0.05$ ).....	38
<b>2- 12.</b> Percentage of vine weevil adults recorded in monitoring tools with openings on the side, top or base - continuous opening or four openings - when released as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test: $p < 0.05$ ).....	39
<b>2- 13.</b> Percentage of vine weevil adults recorded in monitoring tools when testing (A) monitoring tool colour/shade, (B) monitoring tool height and (C) monitoring tool diameter. Vine weevil adults were released as a group of 40 individuals (number of replicate days = 5)(Binomial exact test: $p < 0.05$ ).....	40
<b>2- 14.</b> Percentage of vine weevil adults recorded in monitoring tools when testing (A) monitoring tool density and (B) plant density. Vine weevil adults were released as a group of 40 individuals (number of replicate days = 10). Different letters indicate significant differences between tested monitoring tools (Tukey's HSD test: $p < 0.05$ ); n.s. = not significant.....	41
<b>2- 15.</b> Schematic diagram showing a summary of vine weevil adult preferences to some monitoring tools visual characteristics (colour, height, diameter and entrance positions).....	45
<b>3- 1.</b> Schematic diagram of the Y-tube olfactometer used for two-choice preference tests. Each arm of the olfactometer was connected to an odour source contained in the Drechsel bottle. Air ( $1200 \text{ ml} \cdot \text{min}^{-1}$ ) pumped through a charcoal filter was humidified and blown into each odour Drechsel bottle.....	50
<b>3- 2.</b> Behavioural responses of vine weevil adults (%) toward three experimental treatments: (A) control and control, (B) control and Yew, and (C) control and weevils. Each experimental treatment was tested under three different light conditions: Night (Experiment 1), Day (Experiment 2) and Dawn (Experiment 3). For each experimental treatment a single vine	

weevil adult (number of replicates = 30) was released in the release chamber of the Y- tube olfactometer (binomial exact test: $p < 0.05$ ); n.s. = not significant.....	54
<b>3- 3.</b> Behavioural responses of vine weevil adults (%) toward two experimental treatments: (A) control and yew and (B) control and weevils. Each experimental treatment was tested under three different light conditions: Night (= 🌑, Experiment 1), Day (= 🌞, Experiment 2) and Dawn (= 🌅, Experiment 3) and two photoperiods (Standard and Reverse). For each experimental treatment a single vine weevil adult (number of replicates = 30) was released in the release chamber of the Y- tube olfactometer. n.s., not significant.....	56
<b>3- 4.</b> Percentage of vine weevil adults recorded in monitoring tools under four experimental treatments: (A) yew and control, (B) weevils and control, (C) yew plus weevils and control, and (D) yew plus weevils and yew. For each experimental treatment (carried out under glasshouse conditions) vine weevil adults were released as a group of 40 individuals (number of replicate days = 5) (Binomial exact test: $p < 0.05$ ); n.s., not significant.....	58
<b>4- 1.</b> Gas chromatography/ electroantennogram (GC/EAG) analysis of volatiles from Apple sauce on polar GC column (responses numbered as in Table 1)(Source: Dr Steven Harte, Prof David Hall and Dr Daniel Bray from the University of Greenwich, UK).....	70
<b>4- 2.</b> Behavioural responses of vine weevil adults towards apple sauce at different concentrations 0.1 g (A), 1 g (B), 10 g (C) and 20 g (D) when presented with control in a Y- tube olfactometer. For each experimental treatment 20 vine weevil adults were tested (binomial exact test: $p < 0.05$ ); N.S. = not significant.....	71
<b>4- 3.</b> Behavioural responses of vine weevil adults in a Y-tube olfactometer under four experimental treatments: (A) control and control, (B) control and spindle (20 g) ( <i>E. fortunei</i> ), (C) apple sauce (20 g) and spindle (20 g) and (D) apple sauce (10 g) and spindle (10 g) ( <i>E. fortunei</i> ). For each experimental treatment 20 vine weevil adults were tested (binomial exact test: $p < 0.05$ ); N.S. = not significant.....	72
<b>4- 4.</b> Percentage of vine weevil adults recorded in monitoring tools under seven experimental treatments: (A) apple-vinegar and control, (B) apple-chips and control, (C) apple sauce and control, (D) apple sauce and spindle ( <i>E. fortunei</i> ), (E) apple sauce plus spindle and control, (F) apple sauce plus spindle and spindle and (G) apple sauce plus spindle and apple. For each experimental treatment vine weevil adults were released as a group of 40 individuals (number of replicate days = 5) (binomial exact test: $p < 0.05$ ); N.S. = not significant.....	73
<b>5- 1.</b> Schematic diagram showing the experimental set-up used to determine the toxic effect of a garlic-based product on vine weevil eggs (a) and larvae (b).....	83

<b>5- 2.</b> Schematic diagram showing the experimental set-up used to determine the efficacy of garlic-based product on vine weevil eggs under semi-field conditions.....	89
<b>5- 3.</b> Percentage (mean $\pm$ Std. Err.) of hatched eggs recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) in contact (a), fumigant (b) and combined contact and fumigant (c) tests. Thirty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR <sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR <sup>®</sup> ) and untreated group (Control) (GLMs with a quasi-Poisson or Poisson distribution: *** = $p < 0.001$ ; NS = not significant).....	92
<b>5- 4.</b> Percentage (mean $\pm$ Std. Err.) of hatched eggs recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) at different concentrations (0 %, 0.1 %, 1 %, 10 % and 100 %) in combined contact and fumigant test. Thirty eggs (number of replicates = 5) were used for testing the effect of Pitcher GR <sup>®</sup> on vine weevil. Different letters indicate significant differences between tested Pitcher GR <sup>®</sup> concentrations (GLM with a quasi-Poisson distribution: $p < 0.05$ ).....	93
<b>5- 5.</b> Percentage (mean $\pm$ Std. Err.) of live larvae recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) in contact (a), fumigant (b) and combined contact and fumigant (c) tests. A single larva (number of replicates = 30) was used for testing the effect of Pitcher GR <sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR <sup>®</sup> ) and untreated group (Control) (GLM with a binomial distribution: ** = $p < 0.05$ ; *** = $p < 0.001$ ).....	94
<b>5- 6.</b> Percentage (mean $\pm$ Std. Err.) of larvae eclosion recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) in combined contact and fumigant test with growing media. Thirty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR <sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR <sup>®</sup> ) and untreated group (Control) (GLM with a quasi-Poisson distribution: *** = $p < 0.001$ ).....	95
<b>5- 7.</b> Mean ( $\pm$ Std Err.) of live nematodes recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) in combined contact and fumigant test over the time (Day: 1, 3, 7, 14 and 21). Different letters indicate significant differences between treatments (Pitcher GR <sup>®</sup> , light blue line; Control, black line) and days (GLM with a quasi-Poisson distribution: Tukey's HSD test: $p < 0.05$ ).....	96
<b>5- 8.</b> Percentage (Mean $\pm$ Std. Err.) of live vine weevil larvae recorded when testing the effect of a garlic-based product (Pitcher GR <sup>®</sup> ) in combined contact and fumigant test under glasshouse conditions. Different letters indicate significant differences between different treatments (GLM with a quasi-Poisson distribution; Tukey's HSD test: $p < 0.05$ ).....	97

**5- 9.** Percentage (mean ± Std. Err.) of live larvae recorded when testing the effect of different combinations between a garlic-based product (Pitcher GR®) and an entomopathogenic nematode, *S. kraussei*, in an Efficacy Bioassay. For each experimental unit (Begonia plant) twenty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR® and/or *S. kraussei* on vine weevil. Different letters indicate significant differences between the treatments (GLM with a quasi-Poisson distribution; Tukey’s HSD test:  $p < 0.05$ ). P24 = Begonia with eggs and Pitcher (applied 24hrs after the eggs were applied) no EPNs; P24SK24 = Begonia with eggs and Pitcher and EPNs (applied 24hrs after the eggs were applied); P24SK28 = Begonia with eggs and Pitcher and EPNs (pitcher applied 24hrs after eggs were applied and EPNs applied 28 days after eggs were applied); P28SK28 = Begonia with eggs and Pitcher and EPNs (applied 28 days after the eggs were applied); SK28 = Begonia with eggs and EPNs (applied 28 days after egg were applied) no Pitcher.....97

**6- 1.** Diagram showing the set-up and the experimental units for the survival experiment (a), the dispersal experiment (b) (under laboratory condition) and the efficacy experiment (c) under glasshouse conditions.....111

**6- 2.** The mean (± S.E.) of entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* and *Steinernema kraussei*, recovered from experimental units containing six different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental units (n = 5) nematodes were counted 1 day, 7 days, 14 days, 21 days and 28 days post-application in the Survival Bioassay. Different letters indicate significant differences between treatments (GLM quasi-Poisson distribution: Tukey HSD test:  $p < 0.05$ ).....113

**6- 3.** The mean (± S.E.) of entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* and *Steinernema kraussei*, counted in the bottom segment of experimental units when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit (four segments of PEX pipe) 100 µl of the nematode solutions (500 IJs) was added to corresponding units (n = 20). Different letters indicate significant differences between the treatments (GLM quasi-Poisson distribution: Tukey HSD test:  $p < 0.05$ )..... 114

**6- 4.** The parasitism rate (%) of vine weevil by *H. bacteriophora* and *S. kraussei* when testing in six different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit (four segments of PEX pipe) 100 µl of the nematode solutions (500 IJs) was added to corresponding units (n = 20) (GLM Binomial distribution:  $p < 0.05$ ). N.S. = not significant .....115

**6- 5.** Percentage (mean ± S.E.) of vine weevil larvae recorded from experimental units (Begonia plant) treated with *S. kraussei*, *H. bacteriophora* or without EPN species (Control) when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit 100 ml of the nematode solutions (25000 IJs) was added to corresponding units (number of replicates = 6). Different letters indicate significant differences between the treatments (GLM quasi-Poisson distribution: Tukey HSD test:  $p < 0.05$ ).....116

**6- 6.** Weight (mean ± S.E.) of vine weevil larvae recorded from experimental units (Begonia plant) treated with *S. kraussei*, *H. bacteriophora* or without EPN species (Control) when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). Different letters indicate significant differences between the treatments (Wilcoxon rank sum test  $W < 0.05$ ).....117

**7- 1.** Diagram showing IPM strategies adopted for vine weevil (*Otiorhynchus sulcatus*) control.....130

## List of Tables

<b>3- 1.</b> Experiments testing the effect of olfactory cues on vine weevil monitoring tool performance.....	52
<b>4- 1.</b> Experiments testing the effect of olfactory cues on vine weevil monitoring tool performance.....	68
<b>4- 2.</b> Compounds identified in gas chromatography coupled mass spectrometry (GC-MS) analyses of volatiles from Apple sauce volatile and relative amounts (EAG responses numbered as in Fig. 1).....	69
<b>5- 1.</b> Summary of each application rate of Pitcher GR <sup>®</sup> used for the bioassays carried out under laboratory and glasshouse conditions.....	82
<b>5- 2.</b> Temperature and humidity (means $\pm$ S.E.) recorded in the Combined Contact and Fumigant Toxicity Bioassay under glasshouse conditions.....	89
<b>5- 3.</b> Treatments used for the Efficacy Bioassay using Pitcher GR <sup>®</sup> and the entomopathogenic nematodes, <i>S. kraussej</i> , carried out under semi-field conditions.....	90
<b>6- 1.</b> Chemical and Physical properties of the growing media used for laboratory and glasshouse experiments.....	107
<b>6- 2.</b> Glasshouse temperatures recorded during the Efficacy Bioassay.....	110
<b>6- 3.</b> The mean ( $\pm$ S.E.) of <i>Steinernema kraussej</i> and <i>Heterorhabditis bacteriophora</i> recovered from six different growing media 1 day, 7 days, 14 days, 21 days and 28 days post application. Different letters indicate significant differences between the treatments (Tukey HSD test: $p < 0.05$ ) .....	113
<b>7- 1.</b> Comparative table between IPM strategies available for vine weevil control.....	131

*'Admiranda tibi sevirum spectacula rerum'.*

(Virgil, 1916)

# Chapter 1: General Introduction

## 1. Introduction: Thesis Overview

Vine weevil, *Otiorhynchus sulcatus* (Fabricius, 1775; Coleoptera: Curculionidae) is one of the most economically important pests of soft-fruit and ornamental crops worldwide (Moorhouse *et al.*, 1992; Pope and Roberts, 2022). Adult vine weevils are extremely polyphagous and known to feed on approximately 150 plant species from a wide range of families (e.g., Masaki *et al.*, 1984). Feeding by adults causes characteristic leaf notching aboveground and typically occurs at night while larval feeding occurs belowground on plant root systems (Moorhouse *et al.*, 1992).

A major hurdle in vine weevil management is effective monitoring and timely application of control measures (van Tol *et al.*, 2012; Pope and Roberts, 2022). This is in part due to the nocturnal activity of the adults and the subterranean life history of the larvae, both of which make it challenging to detect this pest within crops. Growers currently monitor for the presence of vine weevil using, for example, night-time crop assessments or plant damage caused by adults feeding on leaves. Identifying infested areas of the crop using these monitoring methods is, however, both time consuming and unreliable.

To implement a truly integrated management strategy for vine weevil it is necessary to first develop a sensitive and reliable monitoring system. Localised and early control of emerging vine weevil adults will help reduce the costs associated with biological control applications, such as entomopathogenic nematodes or area treated with synthetic chemical insecticides, allowing for more effective integration of controls to maintain the pest population below levels that cause economic losses. A number of tool designs have been used for monitoring vine weevil populations (Roberts *et al.*, 2019a). Despite this, the available monitoring tools have been found to lack the required sensitivity and reliability. Roberts *et al.* (2019a) proposed some aspects of trap design such as size, shape and colour of traps that seem to influence vine weevil monitoring tool efficacy. Understanding how these factors contribute to vine weevil behaviour could inform the design of a novel monitoring tool with increased efficacy and reliability.

An effective vine weevil monitoring tool is likely to require the use of a synthetic chemical lure (Roberts *et al.*, 2019a; Pope and Roberts, 2022). The chemical components of a vine weevil lure have been extensively researched for over twenty years, but no commercial product is currently available. As vine weevils reproduce asexually, they do not produce a sex pheromone, but adults display a strong aggregation behaviour (van Tol *et al.*, 2004a; Nakamuta *et al.*, 2005). Despite this, there has been little progress in identifying vine weevil

specific semiochemicals, with previous work on aggregation pheromones not identifying candidate compounds for use in a lure. In the absence of a vine weevil aggregation pheromone, the development of an effective semiochemical lure for vine weevil adults has focused on host-plant volatiles (Roberts *et al.*, 2019b). Indeed, plant odours have been shown to play an important role in host-plant location and aggregation (e.g., feeding-induced release of plant compounds attracting conspecifics). Vine weevil has a preference for odours from specific host plants belonging to the *Euonymus* (Celastrales: Celastraceae) and *Taxus* (Cupressales: Taxaceae) genera (van Tol and Visser, 1998; van Tol *et al.*, 2002). Some compounds, such as (*Z*)-2-pentenol alone and when presented in combination with methyl eugenol, were found to be attractive for adult vine weevils (Roberts *et al.*, 2019b). Furthermore, electroantennography (EAG) recording from adult weevils has shown strong antennal responses to green leaf volatiles, with the strongest responses to (*Z*)-3-hexenol and (*E*)-2-hexenol, but also to a (*Z*)-2-pentenol (van Tol *et al.*, 2002). These compounds are produced by many species of plant belonging to different families and the responses of vine weevil adults are likely to reflect the wide host range of this species. However, it is clear that further studies are required to fully understand the ecology of this species.

Vine weevil management relied for many years on the use of synthetic broad spectrum insecticide sprays (van Tol *et al.*, 2012). It is known that repeated insecticide applications not only risk environmental pollution, but also negatively impact beneficial insect populations (Hladik *et al.*, 2018). Furthermore, operator safety concerns have led to the withdrawal of several synthetic insecticide options from the market (e.g., organophosphates) (Nag and Gite, 2020). Some effective alternatives to synthetic chemical insecticides for controlling vine weevil include the use of bioinsecticides based on plant extracts. It has, for example, been reported that a natural product derived from the oil of the neem tree (*Azadirachta indica* A.Juss; Sapindales: Meliaceae) can negatively impact vine weevil reproduction, decreasing the number of viable eggs and hatch rate (Cowles, 2004). Further research is, however, needed to identify bioinsecticides based on plant extracts for use against this pest and to determine whether botanical bioinsecticides can be effectively included in a vine weevil management strategy. This includes also the application of botanical bioinsecticides and entomopathogenic nematodes (EPNs) as these have been demonstrated to be effective for controlling other insect pests (Otieno *et al.*, 2016).

Much progress has been made in developing alternatives to conventional synthetic insecticides for the control of vine weevil. In particular, research has focused on the use of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF). Standard practice is now to use EPNs, such as *Steinernema kraussei* (Rhabditida: Steinernematidae) or *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) to control vine weevil larvae (Susurluk and Ehlers, 2008; Haukeland and Lola-Luz, 2010). Numerous studies have

assessed the efficacy of EPNs in controlling vine weevil larvae (Ansari and Butt, 2011). These studies have highlighted the importance of the chemical and physical characteristics of the growing media in determining EPN performance (Kung *et al.*, 1991). However, such work has focused on peat-based growing media, which will be banned for domestic use in the UK by 2024 (Department for Environment, Food and Rural Affairs, 2021). Therefore, it is important for research to focus on the alternatives to peat-based growing media and investigate what, if any, this change will have on the efficacy of EPNs in controlling vine weevil larvae.

This thesis is divided into seven chapters:

1. The first chapter provides a review of the literature with respect to vine weevil biology, ecology and control.
2. The second chapter investigates the influence of visual characteristics of monitoring tools on vine weevil behaviour, with the aim of improving the efficacy of these devices.
3. The third chapter investigates the effect of semiochemicals derived from host plant and conspecifics on vine weevil behaviour with the aim of improving monitoring tool efficacy.
4. The fourth chapter investigates the effect of apple volatiles on vine weevil adults with the aim of developing an effective lure for improved vine weevil monitoring.
5. The fifth chapter investigates the effect of a commercially available garlic-based bioinsecticide, Pitcher GR<sup>®</sup>, against vine weevil eggs and larvae under laboratory and glasshouse conditions. Furthermore, the effect of this product on a commercially available biological control of vine weevil, *Steinernema kraussei*, is assessed in order to determine the potential of combining different strategies for vine weevil control.
6. The sixth chapter investigates whether the efficacy of entomopathogenic nematodes against vine weevil larvae is affected by using peat-free growing media. The experiment also investigates the survival, dispersal and virulence of these EPN species in peat free growing media.
7. The seventh chapter provides a general discussion of the work presented in this thesis, to what extent the study aim has been met and provides recommendations for further studies in this area.

## 2. A Review of the Biology, Ecology and Control of Vine Weevil

Vine weevil, *Otiorhynchus sulcatus* (Fabricius, 1775) (Coleoptera: Curculionidae), is a serious economic pest of soft-fruit and ornamental crops worldwide (Pope and Roberts, 2022). Both the adult and larval stages of the vine weevil damage crops. Adult weevils feed on leaves, resulting in characteristic 'notching' along the edge of the leaf (Evenhuis, 1978), which is considered mainly cosmetic damage (Bennison *et al.*, 2018). By contrast, larval feeding on the roots and crown of plant (Smith, 1932; Cowles, 2001) reduces plant vigour and may cause plant death.

Management options for vine weevil populations have historically been based on the use of foliar insecticide sprays (Nielsen *et al.*, 1978; Reding and Persad, 2009; Reding and Ranger, 2011) that target the adults, whereas soil treatments with insecticides were used to target the subterranean larvae (Moorhouse *et al.*, 1992; Long *et al.*, 2000). Reliance on synthetic pesticides for the protection of crops has been associated with undesirable effects on human and environmental health and often leads to reduced efficacy against the target pest (Barzman *et al.*, 2015). It is for these reasons that there is an urgent need to develop effective, safe and economically acceptable approaches to pest management.

Integrated pest management (IPM) is a sustainable, science-based, decision-making process that relies on complementary control strategies (Dara, 2019) to keep populations below economically acceptable levels (Kogan and Bajwa, 1999). An IPM programme seeks to maximise pest control through the use of naturally occurring beneficial organism (i.e., microorganisms, predators and parasites) as well as physical and chemical controls or habitat modification (Bottrell and Schoenly, 2018).

The requirement for IPM adoption to effectively control vine weevil has been known for over 30 years (Moorhouse *et al.*, 1992). While there has been much work to investigate controls that may be used against vine weevil larvae or adults, IPM programmes have only been partially implemented due to a lack of information on the biology of this pest. Current management of vine weevil is heavily reliant on the use of biological controls, such as entomopathogenic fungi (Ansari *et al.*, 2008) and in particular entomopathogenic nematodes (Bruck *et al.*, 2005; Lola-Luz *et al.*, 2005; Ansari and Butt, 2011) both of which target the larval stages of this pest. By contrast, the use of monitoring, forecasting and cultural controls are little used and there is a lack of alternative control options most notably against adult vine weevils.

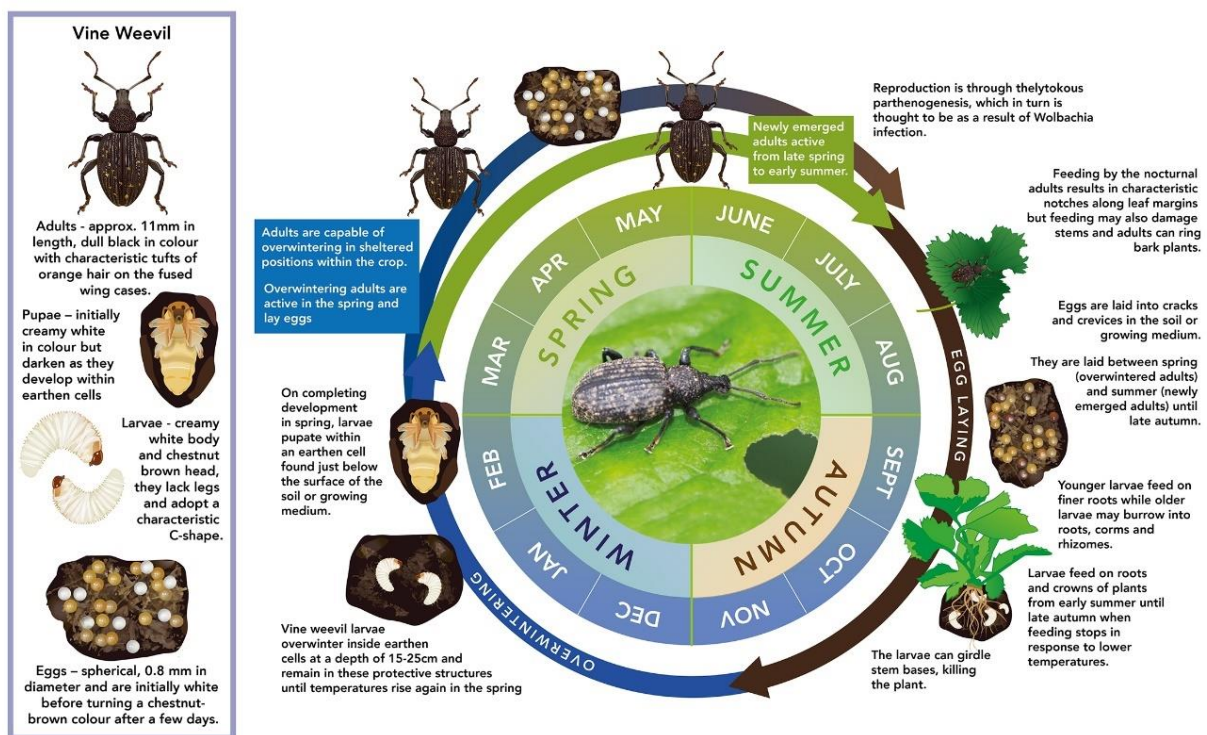
A key feature of this review is to provide a review of vine weevil biology and behaviour and to highlight the available and future strategies that could be adopted to control and limit the damage caused by this pest in economically important crops.

## 2.1 Distribution

Vine weevil is endemic to temperate regions of Europe (Moorhouse *et al.*, 1992). It has expanded its range to North and South America, Central Asia, New Zealand and Japan through plant trade routes (Pope and Roberts, 2022). Currently, vine weevil is one of most economically important insect pests of horticultural and ornamental crops (van Tol *et al.*, 2012; Roberts *et al.*, 2019; Pope and Roberts, 2022).

## 2.2 Life Cycle

Vine weevil development typically takes between nine and eleven months, with most of this time spent as a larva feeding on plant roots (Fig. 1) (Smith, 1932).



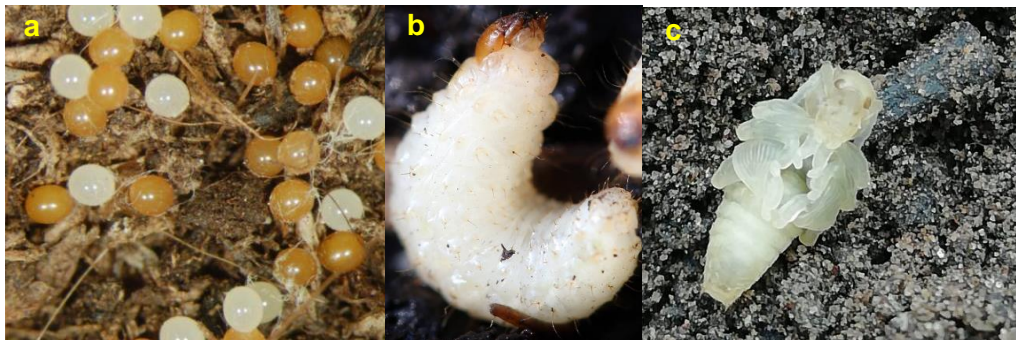
**Figure 1:** Typical life cycle of vine weevil under UK conditions (Source: Penny Greeves).

Generally, there is one generation per year (Moorhouse *et al.*, 1992), but the duration of the different stages is influenced by factors including temperature and humidity (Shanks and Finnigan, 1973; Son and Lewis, 2005b). As such, with no obligatory winter diapause, it is possible that under warm and favourable conditions, such as those found in glasshouses or

polytunnels, the life cycle duration may be shortened (Evenhuis, 1978; Foster, 1982) enabling two generations per year (Schread, 1972).

### 2.2.1 Eggs

Eggs are generally subspherical and 0.8 mm in diameter (Fig. 2a) (Smith, 1932; Foster, 1982). When first laid the eggs are white, but become chestnut brown in colour as they develop (Moorhouse *et al.*, 1992). The time taken for the eggs to hatch is temperature dependent (Stenseth, 1979; Son and Lewis, 2005) and at 20°C egg hatch occurs after around two weeks. Furthermore, egg development is influenced by humidity (Shanks and Finnigan, 1973), however, this has been less often studied.



**Figure 2:** **(a)** Eggs (Source: Bennison *et al.*, 2018), **(b)** larva (Source: author's own) and **(c)** pupa (Source: author's own) of vine weevil.

### 2.2.2 Larvae

All the larval instars are legless, have a chestnut brown head capsule and like most curculionids, a body that forms a characteristic C-shape (Fig. 2b) (Smith, 1932). Larvae may grow to up to 12 mm in length and have a body that is typically creamy white in colour, although larval colour may vary with host plant species (Smith, 1932). The larvae moult between four and nine times, with the number of moults being temperature-dependent (Masaki and Ohto, 1995). Vine weevil larvae develop faster with increasing temperatures up to 24 °C (Son and Lewis, 2005a) and is also thought to be influenced by growing media moisture levels (Shanks and Finnigan, 1973). Larvae continue to feed on plant roots as long as temperatures allow, meaning that while feeding may cease in outdoor situations during the winter, larvae may remain active in protected cropping situation throughout the year (Moorhouse *et al.*, 1992).

### 2.2.3 Pupae

Pupae are creamy white in colour with dusky spines arranged on the head, femora and abdomen (Fig. 2c) (Smith, 1932). The head is bent beneath the body and lies on the breast. Curved hooks characterise the mandibles. Pupae are usually found in cells formed from soil or growing media located near the host plant (Smith, 1932). In the field, pupation generally takes place between mid-May and mid-June (Schread, 1972; Garth and Shanks, 1978), although there are reports where it occurs at other times (Parrella and Keil, 1984). Stenseth (1979) reported that the pupal stage is the most sensitive to temperature among the pre-adult phases, with a relatively high mortality rate during this crucial metamorphic stage. New adults emerge two to three weeks after pupation begins.

### 2.2.4 Adults

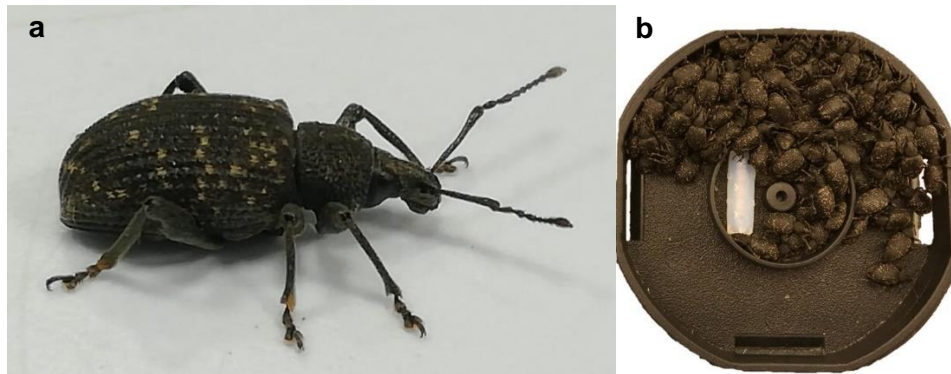
Adult weevils (Fig. 3a) are 8.5-11.5 mm long, grey-black in colour with characteristic tufts of orange hair on their fused elytra (Smith, 1932; Moorhouse *et al.*, 1992) (Fig. 3a). With their elytra fused, adult vine weevils are flightless and within healthy host crops walk only short distances (around 40 cm per day within a favourable strawberry crop) (Pope *et al.*, 2015). However, in response to disturbance or declining host plant quality vine weevil adults are capable of dispersing (over 50 m) to new areas in search of food (Wilcox *et al.*, 1934; Maier, 1978).

Adults are mainly active at night and seek refuge during daylight hours, often aggregating together in groups (Fig. 3b) (Smith, 1932; Moorhouse *et al.*, 1992; Kakizaki, 2001). This aggregation behaviour is thought to be mediated by chemical stimuli (Pickett *et al.*, 1996; van Tol *et al.*, 2004; Nakamuta *et al.*, 2005), however no aggregation pheromone has been identified.

All vine weevils are female (Feytaud, 1918; Smith, 1932), with reproduction through thelytokous parthenogenesis (Lundmark, 2010). This means that new populations can establish from a single weevil (Lundmark, 2010). Eggs (Fig. 1) are laid at varying depths below the soil or growing media surface (Garth and Shanks, 1978). Each female is capable of laying more than 3,000 eggs during its lifetime (Casteels *et al.*, 1994), but between 270 and 450 eggs per female is more common under field conditions (Cram, 1965).

Adult vine weevils are able to persist through the winter by entering a state of dormancy (Moorhouse *et al.*, 2003) or reduced activity, sheltering in protected environments such as soil, leaf litter, or under plant debris. This behaviour helps them survive low temperatures. As spring approaches and temperatures rise, they become active again and start egg laying in spring,

before newly emerged adults from overwintered larvae (Blackshaw, 1966; Garth and Shanks, 1978; Moorhouse *et al.*, 1993). Overwintered individuals may contribute more than half of all vine weevil eggs laid in a season (Blackshaw, 1966).



**Figure 3:** **a)** Adult vine weevil; **b)** Typical intraspecific aggregation between vine weevil adults observed in laboratory condition (Source: author's own and Dr Joe Roberts, respectively).

Newly emerged adults complete a pre-oviposition period of four to nine weeks in May and June with egg laying from June onwards (Bennison *et al.*, 2016; 2018), which continues for as long as conditions remain favourable, often until October and November (Moorhouse *et al.*, 1992).

Preoviposition period, fecundity, egg viability and development have shown to be influenced by the host plant (Cram and Pearson, 1965; Penman and Scott, 1976; Shanks, 1980; Maier, 1981; Cowles, 2004). For example, in blackcurrant, feeding and oviposition have been positively correlated with foliar phosphorus and potassium concentrations and negatively correlated with foliar carbon and zinc concentrations (Clark *et al.*, 2012). Other factors, such as temperature (e.g., Son and Lewis, 2005a), photoperiod (Garth and Shanks, 1978; Nielsen and Dunlap, 1981) and moisture levels (Cram, 1965; Shanks, 1980) have all been suggested to influence oviposition.

### 2.3 Host Range

Host range has been reported to include over 150 species from more than 45 different plant families (Warner and Negley, 1976; Masaki *et al.*, 1984; Moorhouse *et al.*, 1992). In Europe, the host-plant range is believed to be limited to one Gymnosperm genus (*Taxus spp.*)

and a broad range of Angiosperm belonging to two subclasses of the Dicotyledonae, namely Dilleniidae and Rosidae (van Tol *et al.*, 2004). In particular, the families of Primulaceae and Ericaceae (subclass Dilleniidae) and at least six families of the subclass Rosidae (Vitaceae, Celastraceae, Hydrangeaceae, Grossulariaceae, Saxifragaceae and Rosaceae) have been reported to contain many suitable plant species for this weevil (van Tol *et al.*, 2004). Many weed species such as mallow (*Malva* spp.), fat hen (*Chenopodium album*), dock (*Rumex* spp.), knotweeds (*Polygonum* spp.), orache (*Atriplex* spp.), dandelion (*Taraxacum* spp.), rosebay willow herb (*Epilobium* spp.) and plantain (*Plantago* spp.) are also suitable hosts (Masaki *et al.*, 1984; Buxton and Pope, 2011).

## 2.4 Economic Importance

Vine weevil adults feed mainly on above-ground leaves of plants, producing characteristic notches along the leaf edge (Evenhuis, 1978). While this notching has little impact on plant health, it can significantly reduce the economic value of ornamental crops where plant aesthetics are important (Bennison *et al.*, 2018). Larvae negatively impact plant health by feeding on plant roots, corms, rhizomes and subterranean stem tissues, which reduces water and nutrient transport to above-ground plant tissues (Schread, 1972; Penman and Scott, 1976; Moorhouse *et al.*, 1992). Damage caused by vine weevil larvae can lead to significant and even complete crop loss if not effectively controlled (Watt *et al.*, 1999; Pope and Roberts, 2022).

The first documented instance of injury to cultivated plants caused by the larvae and adults of vine weevil was recorded in Germany in 1834 (Smith, 1932). Since then, reports of this weevil damaging economically important plants have become increasingly frequent. The pest has been noted not only across continental Europe, England and Ireland but also in regions such as the United States, Canada and Australia. In many cases, infestations arise from a single weevil walking into the crop or being transported along plant trade routes in pots, as larvae, or in vegetation or other horticultural materials, as adults (Bennison *et al.*, 2018). To date, there is a lack of data to indicate the financial impacts of vine weevil. However, in the UK it has been estimated that more than 2,000 ha of strawberry crops were affected in 2016 with losses of around £ 14 million (Wynn, 2010) and causing up to £30 million worth of damage in the UK ornamentals market (Horgan, 2004).

Factors that may influence crop damage due to vine weevil include growing media type with peat and sand increasing larval survival (Wilcox *et al.*, 1934). In addition, Evenhuis (1978) reported that the position of the larvae within the root system or in the plant crown will also affect how damaging this pest can be. For example, one larva at the base of the stem or in

the plant crown will cause more damage than several larvae around the periphery of the root system. Furthermore, established crops are generally more resistant to weevil damage compared to younger plants and newly transplanted cuttings, as their larger root systems can tolerate feeding damage without affecting growth (Moorhouse *et al.*, 1992).

The continued importance of vine weevil as a pest of soft fruit and ornamental crops has been attributed to reduced availability of persistent pesticides (Cross *et al.*, 1995; Gordon *et al.*, 2006; Gordon, 2008; Wynn, 2010; Clark *et al.*, 2012), changes in crop management practices (Moorhouse *et al.*, 1992), climate change (Tuovinen and Lindqvist, 2009; Johnson *et al.*, 2011), the expansion of horticultural production, increased continental trade of container grown plants, increase in protected cropping systems, peat substrate use and the use of black polythene mulches (Stenseth, 1979; Moorhouse *et al.*, 1992; Watt *et al.*, 1999). Use of peat growing media and polythene mulches in particular provide moist and warm conditions that are thought to favour larval development and adult survival during winter (Bennison *et al.*, 2018) as well as offer protection from predators (Moorhouse *et al.*, 1992).

## 2.5 Endosymbionts

Endosymbiotic bacteria from the *Wolbachia* genus have been detected in a wide variety of invertebrates and may manipulate reproduction in the host through cytoplasmic incompatibility (Sinkins *et al.*, 1995; Zabalou *et al.*, 2004), feminisation (Hurst *et al.*, 1997; Fialho and Stevens, 2000), parthenogenesis (Rodriguero *et al.*, 2010; Mazur *et al.*, 2016) and complete dependence on bacteria for oogenesis and egg production (Dedeine *et al.*, 2001; Stevens *et al.*, 2001). *Wolbachia* infections have been detected in vine weevil (Morera-Margarit *et al.*, 2019), where it is thought to influence egg viability. This has been determined by exposing vine weevil to high temperatures (Son and Lewis, 2005a) or by treating pre-ovipositional females to tetracycline (Son *et al.*, 2008).

## 2.6 Integrated Vine Weevil Management

Integrated Pest Management (IPM) is “a holistic ‘approach’ or ‘strategy’ to combat plant pests and diseases using all available methods, while minimizing applications of chemical pesticides” (Stenberg, 2017). This approach to pest management has been incorporated into a framework Directive 2009/128/EC by the European Union with the aim to achieve sustainable use of pesticides. The intention here is to reduce the risks and impacts of pesticide use on human health and environment as well as promoting the use of alternative approaches

or techniques to conventional use of synthetic pesticides. IPM-based programmes involve the use of preventative methods, the regular monitoring of pests and natural enemies, the use of thresholds for decisions and the simultaneous management and integration of tactics (Kogan, 1998).

### **2.6.1 Monitoring**

A key aspect of any effective IPM programme is the careful monitoring of pest numbers in relation to action thresholds (Kogan, 1998). Detection of vine weevil is made difficult by the nocturnal activity of the adults and the fact that the larvae feed below ground (Moorhouse *et al.*, 1992). This often means that growers only observe this pest within crops after economically significant damage has occurred (van Tol *et al.*, 2012). To be effective, monitoring techniques should detect low numbers of the pest in the crop, determine population dynamics and spatial distribution, detect when a critical threshold for treatment has been reached and determine the timing and effects of control measures (Hanula, 1990; van Tol *et al.*, 2020). If these requirements are met then the monitoring system will help to reduce treatment costs, the risk of crop rejection and increases the value of nursery plants to consumers (Mankin and Fisher, 2002).

#### **2.6.1.1 Detection of Larvae**

The presence of larvae is often seen when plants become stressed and show symptoms of reduced vigour or senescence. Leaves of infested strawberry plants, for example, can turn orange or red in colour (Bennison *et al.*, 2018). However, such observations typically occur after economically significant damage to the crop has occurred. The presence of earlier infestation is more typically detected by “knocking out” the pot and examining the root ball for larvae (Bennison *et al.*, 2016). While effective, this approach can damage plants, is time consuming and may miss infested plants (Mankin and Fisher, 2002). To overcome these limitations a portable acoustic system has been developed to detect the presence and activity of vine weevil larvae (Mankin and Fisher, 2002). However, while the use of acoustic monitoring techniques shows considerable promise for rapid and continuous monitoring of larvae, this approach has yet to be developed for commercial application.

### **2.6.1.2 Directly Counting of Adults**

The presence of adults can be determined through night-time assessments completed using a torch (Bennison *et al.*, 2016; 2018), when weevils are observed on the plants, or by shaking or tapping plants in order to dislodge weevils and observe these individuals on sheets placed beneath plants (Cowles, 1995; Li *et al.*, 1995). While these approaches may provide useful information, they are time consuming, must be completed outside normal working hours and may risk damage to the crop (Cowles, 1995).

### **2.6.1.3 Indicator Plants**

Adult feeding activity in a crop can be detected during daylight hours by looking for the characteristic notches along the leaf margin (Bennison *et al.*, 2016; 2018). While this approach may be effective early in a growing season, as the season progresses, fresh notches become difficult to distinguish from old ones. To overcome this problem plants known to be susceptible to adults can be placed within crops as indicators with which to gauge pest activity and distribution (Berlinger *et al.*, 1996). Alfalfa (*Medicago* spp.) (Wilcox *et al.*, 1934), Himalayan blackberry (*Rubus armeniacus*), salal (*Gaulthoria shallon*) (Cram and Pearson, 1965), *Epilobium* spp. (Cowles, 1995), *Euonymus* spp. and *Primula* spp. (Smith, 1932; van Tol *et al.*, 2004; Bennison *et al.*, 2018) have all been considered for this purpose. However, feeding damage is not always easily seen and it is important that any previous leaf damage to indicator plants is removed so that new notching can be easily seen (Bennison *et al.*, 2016).

Indicator plants can also be used to detect the start of egg laying by adding a layer of sharp sand to the top of the growing medium in which the plants are grown. The sand can be removed at weekly intervals and the eggs, if present, can be floated off in a saturated salt solution (Blackshaw, 1966; Buxton, 2003). However, this monitoring system is time consuming and rarely used in commercial situations.

### **2.6.1.4 Monitoring Tools**

Several studies have investigated the potential of using artificial refuges and traps, hereafter simply referred to as monitoring tools, in order to detect the presence of vine weevil adults within crop environments (Maier, 1983; Hanula, 1990; Li *et al.*, 1995; Gordon *et al.*, 2003). For example, pitfall traps, which involve placing a plastic cup into the ground, have been used to detect vine weevil adults (Casteels *et al.*, 1995). Similarly, methods such as wrapping corrugated cardboard around the stems of larger bushes or trees (Phillips, 1989),

using grooved boards placed on the ground within crops (Li *et al.*, 1995; Gordon *et al.*, 2003) or simple plastic crawling insect traps (Pope *et al.*, 2018) exploited the vine weevil's tendency to seek out shelter during the day. Despite this, most monitoring tool designs tested so far have proved not to be sufficiently sensitive and reliable (Roberts *et al.*, 2019a). For example, Hanula (1990) found that pitfall traps were effective, whereas Maier (1983) showed that grooved boards were more effective than pitfall traps. Roberts *et al.* (2019a) testing different monitoring tool designs under semi-field conditions reported that a black cone shaped design (ChemTica, Costa Rica) was significantly more effective than either grooved boards or pitfall traps. van Tol *et al.* (2020), testing a 'WeevilGrip' fabric 'ruffle' design (Agri-Gripping, The Netherlands), reported that this design detected four to five times more weevils than a grooved board refuge under field conditions. The lack of sensitivity and reliability of monitoring tools, so far tested, in part reflects a lack of understanding of the visual responses of vine weevil adults. Roberts *et al.* (2019a) suggested that the efficacy of designs tested may be influenced by the size, colour, shape or the number and design of the entrances.

#### **2.6.1.5 Semiochemical Lures**

Development of an effective lure would improve the reliability and sensitivity of monitoring tools and create opportunities to develop novel control methods, such as mass trapping as well as lure and kill (Pope *et al.*, 2018). The presence of semiochemicals that could be used in a vine weevil lure is, however, limited by the fact that adults reproduce parthenogenetically and so do not produce a sex pheromone (van Tol *et al.*, 2002). Vine weevil adults do, however, display a strong aggregation behaviour under field and laboratory conditions (Pickett *et al.*, 1996; Kakizaki, 2001; van Tol *et al.*, 2004). Despite these observations, the source and identification of this putative aggregation pheromone has yet to be determined. For example, van Tol *et al.* (2004) and Nakamuta *et al.* (2005) reported attraction between conspecifics while Pickett *et al.* (1996) showed that previously occupied monitoring tools stimulate aggregation behaviour. Pickett *et al.* (1996) noted that three compounds, heptanoic acid, vanillin (4-hydroxy-3-methoxybenzaldehyde) and hexanoic acid elicited the largest electrophysiological responses in vine weevil adults. Despite this, the role of these compounds, if any, in explaining the observed aggregation behaviour of this species is not known.

Most work investigating development of vine weevil lures has focused on volatile organic compounds (VOCs) produced by host plants. Adult weevils have been shown to respond to a wide range of plant odours (Pickett *et al.*, 1996; van Tol and Visser, 1998; van Tol *et al.*, 2002; 2004; Roberts *et al.*, 2019b). For example, Roberts *et al.* (2019a) showed that

vine weevil adults tend to prefer monitoring tools baited with foliage from spindle (*Euonymus fortunei* (Turcz.) Hand.-Maz; Celastrales: Celastraceae) or yew (*Taxus baccata* L.; Cupressaceae: Taxaceae) over unbaited monitoring tools, but this preference is influenced by the adults' prior feeding experience. It remains still unclear how vine weevil adults are able to distinguish between the odours of potential host plants (van Tol and Visser, 2002; van Tol *et al.*, 2012). With such a wide host range (Masaki *et al.*, 1984), it seems likely that the ratios of plant volatiles is important in determining behavioural responses to potential hosts (Bruce and Pickett, 2011). Despite this, vine weevil adults have been reported to respond positively to individual VOCs such as (*Z*)-2-pentenol, 1-hexanol and methyl eugenol (van Tol *et al.*, 2012; Roberts *et al.*, 2019a; van Tol *et al.*, 2020).

Developing an effective lure will need to be combined with the design of a monitoring tool. Currently, little is understood about the required design features of vine weevil monitoring tool. This lack of understanding is illustrated by the fact that van Tol *et al.* (2012) reported that a lure consisting of methyl eugenol and (*Z*)-2-pentenol increased numbers of vine weevil adults close to, but not inside the baited monitoring tool, a boll weevil (*Anthonomus grandis*) trap. By contrast, a lure consisting of just (*Z*)-2-pentenol used together with the 'WeevilGrip' fabric 'ruffle' was found to increase catches of adults in the monitoring tool itself (van Tol *et al.*, 2020).

## 2.6.2 Forecasting

Mathematical models based on knowledge of a pest biology in relation to environmental variables can be used as effective decision support tools (Páez-Chávez *et al.*, 2018). In this way models used to provide forecasts of the pest population can be used to inform pest management decision-making (Tang and Cheke, 2008; Prasad and Prabhakar, 2012) and to predict the impact of different control tactics upon population of the pests (Barzman *et al.*, 2015; Donatelli *et al.*, 2017). Despite the potential of forecasting systems, there has been comparatively little progress in developing a predictive model for the management of vine weevil. This is despite the fact that the effects of environmental factors such as temperature on vine weevil biology are well understood (e.g., Son and Lewis, 2005a, b). Even where models exist, such as a temperature-dependent development rate and survival models (Son and Lewis, 2005a, b) or a population model (Morgan, 1996), these have not been adopted for commercial use. This is due to the fact that these models are derived from laboratory studies carried out under constant temperature conditions, which do not accurately represent the fluctuating environmental conditions that vine weevils experience in the field (Pope and Roberts, 2022). In addition, it would be useful to have a summary model for

complete development from egg to reproductive adult, which can be used to better time applications of controls or to plan within-crop monitoring activities (Pope and Roberts, 2022).

### **2.6.3 Thresholds**

Within an IPM programme there is typically an economic justification for any crop protection measures taken. Key to this is the economic (or action) threshold (ET), which represents the point where a management practice should be implemented to prevent that pest population from causing economically significant reduction in crop value (Stern, 1973; Higley and Pedigo, 1993; Naranjo and Ellsworth, 2009). The ET can be determined through research or a past experience (Nault and Shelton, 2010).

Economic thresholds for vine weevil have focused on the numbers of larvae rather than the number of adults and have been calculated either per plant (Penman and Scott, 1976; LaLone and Clarke, 1981) or unit area of crop (Booth *et al.*, 2002). Fixed ETs, such as these, are usually developed when pest population dynamics are poorly understood and ignore variables, such as population growth and crop damage (Pedigo, 1996). While current ETs are useful as a basis for making pest management decisions these could be greatly improved through development of dynamic ETs that take account of changes in pest life cycle and crop growth stages (Riley, 2008). For vine weevil, the development of reliable ETs may become possible if improved monitoring techniques to detect this species in the field can be developed and both the injurious and non-injurious life cycle stages can be considered (Pope and Roberts, 2022).

### **2.6.4 Prevention**

Prevention, often referred to as cultural controls, is an important feature of IPM programmes (Glen, 2000; Sexson and Wyman, 2005; de Graaf *et al.*, 2008; Rendon *et al.*, 2020). Preventative measures for vine weevil include use of crop rotation (Wilcox *et al.*, 1934), where a new crop is planted at least 100 meters from an infested crop (Moorhouse *et al.*, 1992), disposal of old substrates and plant material (Bennison *et al.*, 2018), control of weeds within and around crops (Bennison *et al.*, 2016), use of cover crops such as white clover (Cone, 1963; Penman and Scott, 1976), the removal of foliage and fruits after harvest to disrupt oviposition (Garth and Shanks, 1978) and use of physical barriers such as sticky bands on the stems of shrubs (Garth and Shanks, 1978; Antonelli and Campbell, 1981; Cowles, 2003) and row covers (Watt *et al.*, 1999).

Use of host plant resistance is a preventive measure that is used in many IPM programmes (Sharma and Ortiz, 2002; Kiggundu *et al.*, 2003; Stout and Davis, 2009). In the case of vine weevil, there have been several studies that have considered the potential of exploiting genetic resources present in strawberry species (Cram, 1980; Shanks *et al.*, 1984; Shanks and Doss, 1986; Doss *et al.*, 1987, 1991; Doss and Shanks, 1988; Labuschagne *et al.*, 1997). Much of this work has focused on clones of beach strawberry (*Fragaria chiloensis* (L.) Mill.; Rosales: Rosaceae) where resistance has been linked to a dense covering of simple hairs on the abaxial leaf surface of leaves (Nielsen *et al.*, 1978; Doss, 1983; Shanks *et al.*, 1984; Shanks and Doss, 1986; Doss *et al.*, 1987). Similarly, leaf characteristics are also thought to be important in reported resistance of lepidote *Rhododendron* (Ericales: Ericaceae) spp. to vine weevil. Here, resistance is thought to be conferred through the scales and associated essential oils found on the leaves of these plants (Doss, 1983; 1984). Despite this, no conventionally bred varieties of soft fruit or ornamental crops have been developed with resistance to this pest. Similarly, no transgenic crops with resistance to vine weevil have been produced despite work demonstrating the potential of cowpea protease trypsin inhibitor (*CpTi*) (Graham *et al.*, 1997; 2002).

## **2.6.5 Control**

### **2.6.5.1 Synthetic Chemical Insecticides**

Synthetic chemical insecticides have not been used in commercial situations against vine weevil eggs. Despite this, insecticides such as chitin inhibitors (e.g., lufenuron) and growth regulators (e.g., diflubenzuron) have been shown to be effective against eggs (Sol, 1985; Jay and Cross, 2000; Reineke and Hauck, 2012).

Larval stages have more often been the target of insecticides applications. Historically, persistent organochlorine insecticides, such as aldrin were used (Cone, 1963) and more recently organophosphate (Blackshaw, 1987; Cross *et al.*, 1995) (e.g., chlorpyrifos), neonicotinoids (e.g., imidacloprid) (Parsons *et al.*, 1988; Cowles, 2001; Reding and Persad, 2009) and pyrethroids (e.g., bifenthrin) (Kepler and Bruck, 2006). Typically, these insecticides were incorporated into the growing medium or used as dust (Cram and Andison, 1959; Mason, 1960), but drenches (e.g., chlorpyrifos) have also been used (Cowles, 2003).

Vine weevil adults have typically been controlled through the use of foliar applications of synthetic chemical insecticides (Cowles, 1995) including organophosphates, carbamates, and pyrethroids (Blackshaw and O'Neill, 1987; Watt *et al.*, 1999; Reding and Persad, 2009; Reding and Ranger, 2011). Insecticides targeted against adults have typically been applied during the preoviposition period of newly emerged adults (Hanula, 1990; van Tol *et al.*, 2012).

Approval for the use of synthetic insecticides for vine weevil control has been withdrawn due to the negative impacts of these products on human health (e.g., chlorpyrifos) (EFSA, 2019) and the environment (e.g., imidacloprid and fipronil) (Godfray *et al.*, 2014; Holder *et al.*, 2018). This has meant that there are currently no synthetic insecticides approved for the control of vine weevil larvae or adults in the UK.

### **2.6.5.2 Bioprotectants**

Bioprotectants are used to protect against unwanted organisms including pests and pathogens (IBMA, 2018). A bioprotectant should originate from nature and/or be 'nature identical' if synthesised. Bioprotectants include invertebrate biocontrol agents or macrobials (e.g., insect, mite and nematodes species), microbials (bacteria, fungi, oomycetes, viruses and protozoa), natural substance (e.g. substances that may be derived from plants, algae/micro algae, animals, minerals, bacteria, fungi, protozoans, viruses, viroids and mycoplasmas), semiochemicals (e.g., substances emitted by plants, animals and other organisms used for intra- species and/or inter-species communication) (IBMA, 2018). The terms bioprotectant and biopesticide are often used interchangeably. However, the term bioprotectant refers only to the use of micro-organisms and natural products sold for the control of plant pests (Chandler *et al.*, 2011). Today bioprotectants have become key components of many IPM programmes due, in part, to their safety to operators short harvest intervals, lack of pest resistance to these products, compatibility of these products with other pest management strategies such as natural enemies, resistant varieties, *etc.*, allowing, in theory at least, additive or synergistic interactions that benefit pest management (Copping and Menn, 2000; Srinivasan, 2012).

#### **2.6.5.2.1 Microbial Bioprotectants**

Entomopathogenic fungi (EPF) have been the focus of several studies to improve control of vine weevil (Moorhouse *et al.*, 1993; Lola-Luz and Downes, 2007; Shah *et al.*, 2007; Haukeland and Lola-Luz, 2010). In particular, *Metarhizium brunneum* (= *M. anisopliae* (Metchnikoff) Sorokin; Hypocreales: Clavicipitaceae) and *Beauveria* (Hypocreales: Cordycipitaceae) spp. have been shown to be effective in controlling vine weevil larvae (Easterbrook *et al.*, 1992; Zimmermann, 1992; Booth and Shanks, 1998; Bruck and Donahue, 2007; Ansari and Butt, 2011; Rondot and Reineke, 2017; Noble *et al.*, 2018). Although vine weevil larvae are reported to be more susceptible to fungal infection than adults (Moorhouse

*et al.*, 1993), Pope *et al.* (2018) demonstrated the effectiveness of using a novel control system based on autodissemination of EPF for weevil adults.

*Metarhizium brunneum* (= *M. anisopliae*) isolate V275 (F52 = BIPESCO 5), was sold as a granular bioinsecticide (Met52 Granular Bioinsecticide<sup>®</sup>, Novozymes, Denmark) in the UK for the control of vine weevil, however this product is no longer commercially available. More recently, a new product (Lalguard M52<sup>GR</sup>), which can be used within growing media for the control of vine weevil, has been launched by ICL Specialty Fertilizers containing *Metarhizium brunneum* strain Ma43 and has been made commercially available in the UK and Ireland.

Ansari and Butt (2013) reported that *M. brunneum* can provide 71-96 % control of vine weevil larvae when applied as a granular mix into a soil-less media. However, EPF are ectothermic organisms, meaning that their activity is influenced by temperature (Ansari and Butt, 2013; Klingen *et al.*, 2015). While isolates of *B. bassiana*, *B. pseudobassiana* and *M. brunneum* have been shown to infect and kill vine weevil larvae at low temperatures (Klingen *et al.*, 2015), *M. brunneum* V275 is most effective at 20-25 °C (van Tol, 1993; Moorhouse *et al.*, 1994; Bruck and Donahue, 2007). Therefore, application timing is critical in many northern hemisphere regions to achieve effective control. Furthermore, although Met52<sup>®</sup> was used in all types of soil and growing media, efficacy where crops were grown in heavy soil types was reduced (Bennison *et al.*, 2018).

In addition to their role as microbial biopesticides, many endophytic EPF species can colonize internal plant tissues without causing any noticeable symptoms of disease (Wilson, 1995; Vega, 2018). This colonization has been reported to be beneficial for the plant as endophytic EPF species act not only as pest and disease control agents in many economically important crops but also as plant growth promoters (Mantzoukas and Eliopoulos, 2020). In the case of vine weevil control, Rondot and Reineke (2017) showed that a foliar application of a *B. bassiana* (Naturalis<sup>®</sup>, CBC (Europe) S.r.l. – BIOGARD Division) on young potted grapevine plants deterred this pest and indicated a new mode of action of plant-associated entomopathogenic fungi for pest management programmes.

Similar to the endophytic EPF species, arbuscular mycorrhizal fungi may also influence interactions between plants and insect herbivores, often enabling the plant to resist or tolerate herbivore attack (Vannette and Hunter, 2009; Hage-Ahmed *et al.*, 2019). In the case of vine weevil, the presence of arbuscular mycorrhiza (e.g., *Glomus mosseae*, *G. fasciculatum*; Glomerales: Glomeraceae) has been shown to reduce the survival (e.g., 48-55 %) and size (e.g., 37 %) of vine weevil larvae feeding on the roots of plants (Gange *et al.*, 1994; Gange, 1996; 2001).

Some species of bacteria, known as entomopathogenic bacteria (EPB) have evolved strategies to invade the immune responses and kill host insects (Ruiu, 2015). Among these

species of EPB, *Bacillus thuringiensis* (*Bt*, Bacillales; Bacillaceae) is the most widely studied and used (Reede *et al.*, 1985; Kaya *et al.*, 1995; Dias *et al.*, 2005; Sanahuja *et al.*, 2011). Despite this, comparatively little work has investigated the potential of *Bt* against vine weevil and there are no commercially available products for this use. Work that has been done has shown the *Bt* subsp. *kurstaki* used as a substrate-incorporated treatment for control vine weevil larvae on *Primula*, to be effective, killing 83 % of larvae (Blackshaw, 1984). Similarly, *Bt* subsp. *tenebrionis* killed 70 % of larvae under constant temperature conditions of 20 °C but was ineffective against vine weevil adults in protected and field grown crops (Landi, 1990). Despite this promise, there are currently no commercially available products based on *Bt* with approval for use in soil or growing media in the UK.

#### **2.6.5.2.2 Natural Substance Bioprotectants**

Natural substance bioprotectants include plant derived products that are used for the management of insect pests (Pavela, 2016; Lengai *et al.*, 2020). These products typically have good environmental profiles, while target species are unlikely to develop resistance (Shaaya *et al.*, 1997; Dubey *et al.*, 2008; Saroukolai *et al.*, 2010; Gaikwad *et al.*, 2012; Joseph and Sujatha, 2012). The insecticidal activity of botanical natural substance bioprotectants is attributed to the presence of secondary metabolites (Pavela, 2016) that affect basic biochemical processes and consequently the physiology and behaviour of the target insect (Regnault-Roger *et al.*, 2012; Pavela and Benelli, 2016; Isman and Tak, 2017). Typical observable effects include disrupting key physiological processes, such as respiration and moulting as well as exciting the central nervous system and modifying insect behaviour by acting as phago-stimulants, anti-oviposition, antifeedants, deterrents or attractants cues (Isman, 2006; Guleria and Tiku, 2009; Miresmailli and Isman, 2014). For example, when the naturally occurring phenylpropanoid precursor, cinnamamide, was applied to strawberry plants this deterred vine weevil adults from feeding (Mosson *et al.*, 1996).

Oil from the seed of the neem tree (*Azadirachta indica* A. Juss.; Sapindales: Meliaceae) has received the most attention as a plant-derived natural substance bioprotectant for the control of vine weevil. Cowles (2004) showed that a continuous dietary exposure to azadirachtin, a group of compounds found in neem oil, reduced the ability of vine weevil adults to produce eggs and decreased the viability of eggs that were laid. After several weeks feeding on azadirachtin treated leaves, adult mortality increased. Other work has shown that neem also inhibits growth and increases the mortality of vine weevil larvae (Gaffney *et al.*, 2005; Shah *et al.*, 2008). Shah *et al.* (2008) reported that neem seed cake additively enhanced the efficacy of *M. brunneum* (= *M. anisopliae*) for the control of vine weevil larvae in outdoor

potted *Euonymus* plants. A similar study has shown the potential of combining neem with *Beauveria* spp. for the control of vine weevil larvae in potted plants and organic crops (Kowalska, 2008).

Recently, a granular formulation of garlic has been made commercially available for the control of vine weevil eggs and young larvae as part of an IPM programme in containerised ornamental plants (ICL Specialty Fertilizers, 2024). Here, the active ingredient is a range of diallyl polysulfides, which are lethal after passing through the insect cuticle and reacting with low molecular weight thiols. The oxidative stress created by the diallyl polysulfides affects many cellular processes meaning that, as with other natural substance bioprotectants, resistance to this control is unlikely to develop in the pest (Senthil-Nathan, 2015).

### **2.6.5.3 Invertebrate Biological Control Agents**

Biological pest control by natural enemies is an important component of sustainable crop production (Thomson and Hoffmann, 2010; Perez-Alvarez *et al.*, 2019).

Insectivorous mammals such as hedgehogs, moles and shrews have been shown to feed on vine weevil (Feytaud, 1918). Smith (1932) reported predation by deer mice and skunks on this species. Additionally, various bird species, including starlings, tits, finches, flycatchers, and several warblers, have been observed preying on vine weevils in the UK (Bennison *et al.*, 2018). Feytaud (1918) reported that poultry were effective at scavenging both larvae and adults, while small flocks of bantams have also been used to control adult weevils and larvae (Evenhuis, 1978). Feytaud (1918) reported different lizard species act as predators of vine weevils. Frogs and toads were also considered to be very important and effective naturally occurring predators due to their nocturnal foraging activity in vineyards (Smith, 1932). Beetles, including species of Carabidae (Feytaud, 1918) and Staphylinidae (Smith, 1932; Moorhouse *et al.*, 1994) have been recorded to prey on vine weevil eggs, larvae or adults in strawberry and blackcurrant plantations (Bennison *et al.*, 2018). In particular, the European earwig (*Forficula auricularia* L.; Dermaptera: Forficulidae) has been recorded to prey on eggs and larvae (Garth and Shanks, 1978), whereas the rove beetle (*Dalotia coriaria* Kraatz; Coleoptera: Staphylinidae) has been recorded to prey on young vine weevil larvae (Bennison *et al.*, 2016). Other invertebrates that can act as natural enemies of vine weevil include some parasitoids such as *Blacus tuberculatus* (Hymenoptera: Braconidae) (Feytaud, 1918) and tachinid flies (Diptera: Tachinidae) (Thiem, 1922). Spiders have been documented to attack adults of this species, whereas mite predation was observed on eggs (Bennison *et al.*, 2018).

In the last 30 years many studies have investigated the potential of EPNs for the control of vine weevil (Wilson *et al.*, 1999; Bruck *et al.*, 2005; Ansari *et al.*, 2008; Guy *et al.*, 2017). Currently nematode species from Heterorhabditidae and Steinernematidae families are used commercially for the control of vine weevil larvae. Of the *Heterorhabditidae*, *Heterorhabditis bacteriophora* (Poinar) is commonly used in commercial situations in the UK, but *H. downesi* (Stock, Griffin and Burnell) is also available in some regions. Other species of Heterorhabditis have also been reported as being capable of infecting vine weevil including *H. heliothidis* (Khan, Brooks and Hirschmann) (Bedding and Miller, 1981; Scherer, 1987), *H. marelatius* (Liu and Berry) (Berry *et al.*, 1997) and *H. megidis* (Poinar, Jackson and Klein) (Kakouli-Duarte *et al.*, 1997; Long *et al.*, 2000; Lola-Luz and Downes, 2007). Most studies have found that the Heterorhabditis species so far studied provide good control against late instar larvae as well as pupal stages of vine weevil (Bedding *et al.*, 1983; Schirocki and Hague, 1997). Among the *Steinernema* species, several are commercially available for vine weevil control in the UK, including *S. carpocapsae* (Weiser), *S. kraussei* (Steiner) and *S. feltiae* (Felipjev) (Haukeland and Lola-Luz, 2010; Ansari and Butt, 2011a; Guy *et al.*, 2017). Other species have been also reported to infect vine weevil larvae, including *S. riobrave* (Cabanillas, Poinar and Raulston) (Bruck *et al.*, 2005), *S. glaseri* (Steiner) (Georgis and Poinar, 1984; Jackson *et al.*, 1985) and *S. bibionis* (Bovien) (Godliman, 1991). *Steinernema kraussei* is the most commonly used of the Steinernematidae (Long *et al.*, 2000; Willmott *et al.*, 2002; Haukeland and Lola-Luz, 2010) in commercially grown crops as it has been shown to be as or more effective as *H. bacteriophora* and is also effective at lower temperatures.

In early comparative studies *Heterorhabditis* spp. have been reported to be more effective than *Steinernema* spp. (Dorschner *et al.*, 1989; Bruck *et al.*, 2005). However, since *S. kraussei* has been available, this species has been shown to be equally or more effective than different *H. bacteriophora* products in strawberry (Hough *et al.*, 2015) and equally as effective as *H. bacteriophora* on Fuchsia (Bennison, personal communication).

The survival and pathogenicity of EPNs is affected by factors such as temperature, substrate or soil type, moisture as well as density of larvae (Long *et al.*, 2000; van Tol and Raupp, 2006; Stuart *et al.*, 2015). Temperature is critical for nematode activity and each EPN species or strains is only effective within a certain temperature range (Kung *et al.*, 1991; Georgis, 1992; Griffin, 1993). The inefficacy of *Heterorhabditis* spp. at low temperatures is associated with an inhibition of nematodes searching rather than the ability of the EPN to penetrate the larvae (van Tol, 1993). By contrast, the cold-active EPN, *S. kraussei*, remains active and effectively kills vine larvae at soil or growing media temperatures as low as 5 °C (Willmott *et al.*, 2002). This means that *S. kraussei* can be used effectively earlier in the spring and later in the autumn than *H. bacteriophora* for the control of vine weevil. In the UK, growers tend to use *H. bacteriophora* products when substrate or soil temperatures are suitable (12-

30 °C or 14-33 °C depending on product and strain) and switch to *S. kraussei* when temperatures are cooler (suitable temperature range 5-30 °C).

Entomopathogenic nematode survival and movement is affected by substrate or soil type to which they are applied (Kung *et al.*, 1990; Kakouli-Duarte *et al.*, 1997; Koppenhöfer and Fuzy, 2006; Ansari and Butt, 2011). This is because nematodes require a film of water and an open texture to the soil or substrate for movement (Grant and Villani, 2003). As a result, EPN species used as biological controls of vine weevil are less effective in dry and heavy soils such as clay than in substrates such peat (Kung *et al.*, 1990; Haukeland and Lola-Luz, 2010).

Entomopathogenic nematodes are typically applied in August to early September in soft fruit crops in the UK and in late August to October in protected ornamentals, when the majority of vine weevil eggs have been laid, larvae are present and temperatures are still suitable for the product used (Fitters *et al.*, 2001; Lola-Luz and Downes, 2007; Bennison *et al.*, 2016; 2018). Applications may also be made between April and early May to target any larvae which have overwintered but before pupation (Bennison *et al.*, 2016; 2018). A single application of EPNs will in most cases not provide 100 % control and so a repeat application should be considered (Irving *et al.*, 2012; Bennison *et al.*, 2016). In some cases, multiple applications of EPNs are used throughout the season through the irrigation system, using a 'little and often' approach (Bennison *et al.*, 2017; 2018).

EPNs have been found to be most effective for the control of vine weevil larvae when applied to potted plants and crops grown under protection (Bedding and Miller, 1981; Simons, 1981; Dolmans, 1983; Georgis and Poinar, 1984; Rutherford *et al.*, 1987). EPNs are less reliable in soil-grown crops than those grown in growing media as crops grown in growing media are more effectively irrigated than those grown in soil. In addition, under field conditions, soil temperatures are typically lower, which affect EPN efficacy (Jagdale and Gordon, 1998; Lee *et al.*, 2016; El Khoury *et al.*, 2018; Lortkipanidze *et al.*, 2019).

While EPNs are mainly used against vine weevil larvae, Bedding and Miller (1981) reported that *H. heliothidis* was able to infect newly emerged adults. More recently, a gel formulation of *S. carpocapsae* applied to grooved wooden boards has been developed (E-nema, Germany). The EPNs in the gel formulation infect the vine weevil adult as it takes refuge within the grooves of the wooden board, leading up 92 % mortality within 30 days (Bennison *et al.*, 2018).

### **3. Thesis Aims and Objectives**

The aim of this study is to contribute to the development of an effective IPM programme for vine weevil through developing novel IPM-compatible tools that improve both monitoring and control of this pest.

The objectives are:

1. Determine the importance of visual characteristic of vine weevil monitoring tool efficacy.
2. Investigate the potential of semiochemicals from host plants and conspecifics that may be used to improve monitoring tool efficacy.
3. Investigate the potential of semiochemicals from apple-based products that may be used to improve monitoring tool efficacy.
4. Determine the efficacy of a commercially available garlic-based bioinsecticide against vine weevil eggs and larvae.
5. Record the efficacy of entomopathogenic nematodes against vine weevil larvae when applied to peat-free growing media.

# Chapter 2: Effect of Visual Cues on Monitoring Tool Efficacy<sup>1</sup>

## Abstract

Vine weevil remains one of the most economically important insect pests of soft-fruit and ornamental crops globally. Growers currently lack effective monitoring systems to determine presence of vine weevil within crops, meaning that controls are often applied too late to prevent economic losses. Development of improved monitoring systems is currently hindered by a lack of knowledge of whether vine weevil adults select a monitoring tool based on its visual appearance. This study used paper cups as monitoring tool to investigate the behavioural responses of adult vine weevils to visual cues (e.g., monitoring tool shade/colour, entrance position, height and diameter as well as the effect of monitoring tool and plant density) under laboratory and glasshouse conditions. Vine weevil preferences were determined by the number of individuals recorded within a monitoring tool. When provided with a binary choice between black or white monitoring tools, vine weevil adults showed a preference for black monitoring tools under laboratory conditions. Vine weevils provided with a range of coloured monitoring tool (blue, green, red and yellow) in addition to black and white monitoring tools showed a preference for black and blue over the other colours and white monitoring tools in group choice experiments. Monitoring tool height and entrance position also influenced vine weevil behaviour with individuals exhibiting a preference for taller monitoring tools and those with entrance openings around the monitoring tool base. Under glasshouse conditions, monitoring tool colour/shade, height and diameter all influenced monitoring tool efficacy, with individuals exhibiting a preference for black, tall and wide monitoring tools. In addition, the total number of individuals recorded in monitoring tools increased with monitoring tool density.

This study provides information on key factors that influence vine weevil monitoring tool efficacy and can be used to inform the development of a new monitoring tool for this pest. In addition, preliminary information is also given on how crop habitat influences monitoring tool efficacy.

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<sup>1</sup> Sections of the following Chapter have been published in:

1: Fezza *et al.* (2022). Optimising Vine Weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), Monitoring Tool Design. *Insects*, 13, 80. <https://doi.org/10.3390/insects13010080>.

2: Fezza *et al.* (2023). Decoding attraction: Improving vine weevil monitoring by exploiting key sensory cues. *Pest Management Science*, 79 (11), pp. 4635-4643. <https://doi.org/10.1002/ps.7665>.

## 1. Introduction

Effective integrated pest management (IPM) programmes require reliable pest population monitoring techniques to determine when economic thresholds have been exceeded and then implement appropriate control measures (Barzman *et al.*, 2015). Various methods are used to detect the presence of pests and monitor their population growth within crops (e.g., visual assessments of crop), but many approaches rely on dedicated monitoring tool designed for specific pests (Preti *et al.*, 2021). A wide range of monitoring tool designs are used to detect insect pests as a result (Cardim Ferreira Lima *et al.*, 2020), but the efficacy of each tool is determined by an ability to exploit knowledge of the target pest's ecology and behavioural response to visual stimuli.

Vine weevil remains one of the most important pests of horticultural and ornamental crops globally (Pope and Roberts, 2022). This is largely due to the fact that monitoring is challenging as the larvae feed below ground while the adults are nocturnal, therefore controls are often applied too late to prevent economically significant losses (Pope and Roberts, 2022). The importance of correctly timing control applications has increased as vine weevil management has shifted from a reliance on persistent broad-spectrum insecticides to entomopathogenic nematodes (EPNs) and fungi (EPFs) used to target vine weevil larvae (Ansari *et al.*, 2008). Indeed, despite widespread use of such controls, growers continue to report significant crop losses associated with vine weevil infestation and it is likely that this is in part due to the application of controls being incorrectly timed without use of reliable pest monitoring (Pope and Roberts, 2022).

Different monitoring tool designs have been used to detect the presence of vine weevil populations, including grooved wooden boards placed on the ground (Li *et al.*, 1995; Gordon *et al.*, 2003), pitfall traps (Hanula, 1990), corrugated cardboard or ruffled material wrapped around plants (Phillips, 1989; van Tol *et al.*, 2020) and commercially available crawling insect traps (Pope *et al.*, 2018), including one designed specifically for vine weevil (Roberts *et al.*, 2019a). Despite extensive work to develop vine weevil monitoring tools, their efficacy is variable and often cannot be used to confidently monitor vine weevil populations in crops (Roberts *et al.*, 2019a). This lack of sensitivity and reliability reflects, in part, a limited understanding of vine weevil biology and visual ecology, which is reflected in the unreliability of commercially available monitoring tools (Pope and Roberts, 2022).

Visual appearance is important in the design of effective monitoring tools for many insect pests (Coli *et al.*, 1985; Mahot *et al.*, 2020). Colour is often an important design element and the preference for a particular colour is typically species specific and, in some cases, habitat dependent (Mizell and Tedders, 1999). Even for nocturnal species, such as the adult banana root borer (*Cosmopolites sordidus* Germar; Coleoptera: Curculionidae), colour

preference has been reported, in this case to mahogany-brown monitoring tools (Reddy *et al.*, 2009).

Other visual cues used by insects for orientation include size and shape (Reddy *et al.*, 2011; Wang *et al.*, 2019). As a result, monitoring tool size, shape and entrance number/location have been suggested to be key factors in determining the efficacy of monitoring tools (Roberts *et al.*, 2019a). For example, large monitoring tools are reported to be more effective than smaller ones in some weevil species, including the banana root borer (Reddy *et al.*, 2011). Leskey (2006) showed that pyramid-shaped traps are more effective than cylindrical traps for monitoring plum curculio weevil (*Conotrachelus nenuphar* Herbst; Coleoptera: Curculionidae). Furthermore, a direct relationship has been shown between the number of monitoring tool entrance holes and number of rice weevils (*Sitophilus oryzae* L.; Coleoptera: Curculionidae) captured (Epsky and Shuman, 2002). Monitoring tool position and density in relation to plants found in the same environment may also influence the number of individuals interacting with it (Lessio and Alma, 2004).

The present study investigated the role of visual cues in monitoring tool choices made by vine weevil adults under laboratory and glasshouse conditions. Specifically, the study sought to answer the following questions:

- (i) Do shade/colour, entrance position, height and diameter influence the efficacy of vine weevil monitoring tools under laboratory and glasshouse conditions.
- (ii) Is the efficacy of vine weevil monitoring tools influenced by abiotic factors such as monitoring tool or plant density under glasshouse conditions.

It is hypothesised that colour, height, diameter and entrance position of monitoring tools influence the behaviour of adult vine weevils. Furthermore, it is proposed that the total number of vine weevils recorded in the monitoring tools may also be influenced by both the design of the tool and the density of surrounding plants.

## **2. Material and Methods**

### **2.1 Insects**

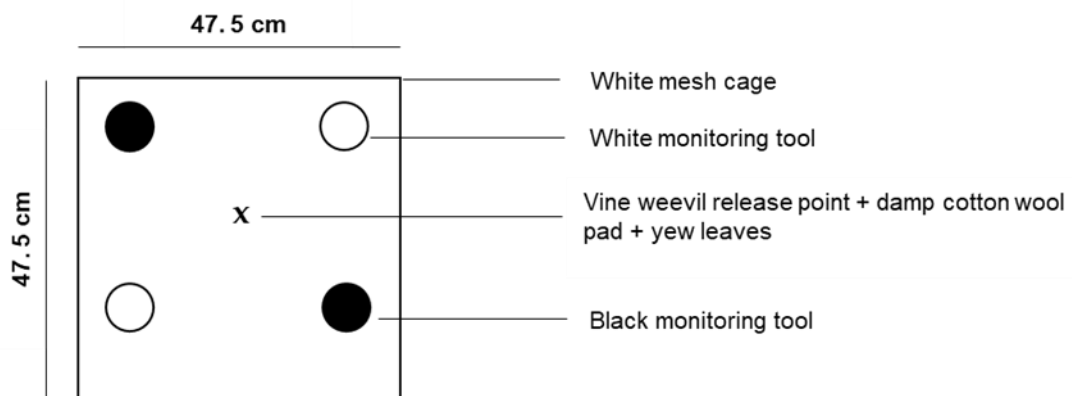
Vine weevil adults were collected from commercial strawberry (*Fragaria × ananassa* Duchesne; Rosales: Rosaceae) crops grown in Staffordshire, UK. These individuals were maintained on yew (*Taxus baccata* L.; Cupressales: Taxaceae) sprigs in plastic terrariums (30 × 19.3 × 20.6 cm; Exo Terra, Castleford, UK) and provided with moist paper towels that were replaced weekly. Thirty-five to forty weevils were placed in each terrarium and housed in a

controlled environment room (20 °C, 60% relative humidity, 16:8 hour light:dark photoperiod; Fitotron, Weiss Technik, Ebbw Vale, Wales, UK) until their use in experiments.

## 2.2 Laboratory Experiments

### 2.2.1 Monitoring Tool Colour (Light vs Dark)

The behavioural response of adult vine weevils to light and dark monitoring tools was tested in a binary-choice experiment investigating individual and group selection under controlled environment conditions (20 °C; 60% relative humidity; 16:8 hours photoperiod). Monitoring tools were constructed from paper cups (height = 7 cm; Ø base = 5 cm; Ø rim = 7 cm) that were externally and internally painted either black or white using poster paint (Galeria Acrylic, Windsor & Newton, London, UK). Paper cups used as monitoring tools were inverted so that the rim became the monitoring tool base and four equally distanced entrances were made in the monitoring tools by cutting 1 cm<sup>2</sup> openings around the cup rim. A roll of corrugated card (length = 30 cm; width = 3 cm) was inserted into each monitoring tool to provide additional shelter. Five experimental arenas were prepared by placing four monitoring tools (two black and two white) in the corners of five white mesh cages (47.5 × 47.5 × 47.5 cm, BugDorm4S4545, MegaView Science Co. Ltd., Taichung, Taiwan) (Fig. 1, 2a).

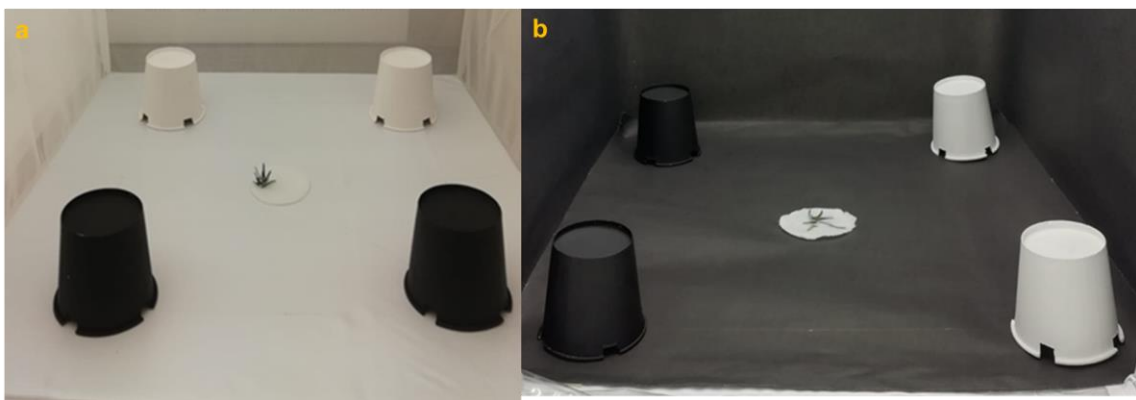


**Figure 1.** Schematic diagram showing the arrangement within each mesh cage for the monitoring tool colour (black vs. white) experiment. Monitoring tool positions are shown in circles and the vine weevil release point using an x mark.

Monitoring tool positions were randomised between replicates to account for potential directional bias. A piece of damp cotton wool and yew leaves were placed in the centre of each cage to provide a source of food and moisture. For the experiment where one weevil adult was released a total of 50 individuals were tested (five replicates per day for ten days) while for the experiment where five vine weevil adults were released a total of 125 individuals

were tested (five replicates per day for five days). As vine weevil adults are nocturnal and seek refuge as dawn approaches, individuals were released three hours prior to the controlled environment room lights switching off at 22:00. Controlled environment room lighting remained off until 06:00 and the position of each vine weevil was recorded at 07:00, providing a one-hour period for vine weevil to seek refuge before an assessment began.

An additional experiment was carried out to determine the effect of background colour on vine weevil preference for black or white monitoring tools. This experiment was carried out as previously described (testing both individual and group selection) but the cage floor and sides were covered with black paper (Clairefontaine Coloured Kraft Roll, ExaClair, King's Lynn, UK) (Fig. 2a, b).



**Figure 2:** Arrangement within each mesh cage for the monitoring tool colour (black vs. white) experiment with different colour backgrounds: white (a) or black (b).

### 2.2.2 Monitoring Tool Colour (Multiple)

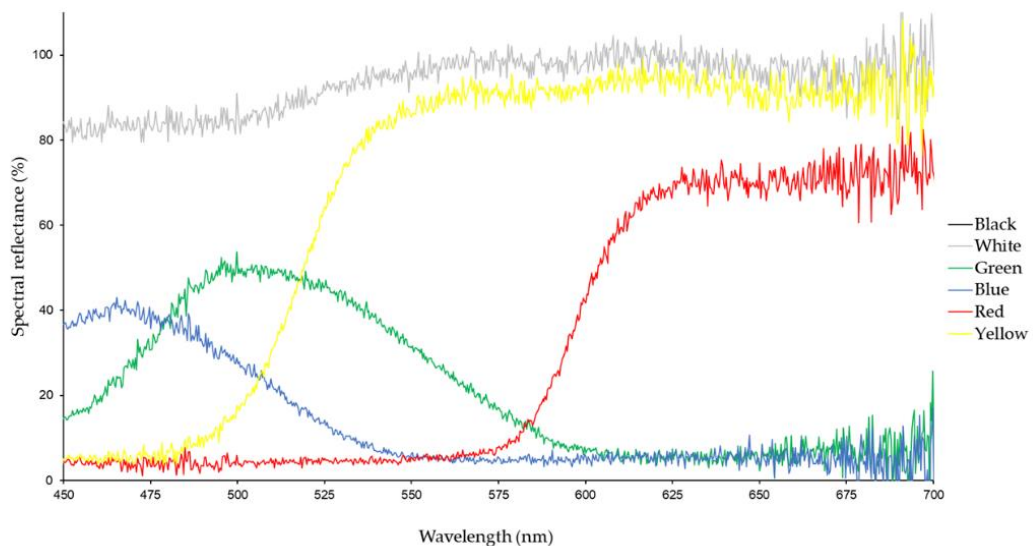
The behavioural response of adult vine weevils to differently coloured monitoring tools (blue, green, red or yellow), as well as black and white monitoring tools, were tested in a six-choice experiment under the same controlled environment conditions as described previously. Monitoring tools were created from paper cups (height = 7 cm;  $\varnothing$  base = 5 cm;  $\varnothing$  rim = 7 cm) and painted one of four colours or black or white using poster paint (Fig. 3). Refuge colour was determined using reflectance measurements acquired using a spectrophotometer (Flame Miniature Spectrometer, Ocean Insight, Duiven, The Netherlands) with a spectral wavelength range from 450 to 700 nm (Fig. 4). Paper cups were inverted as previously described and four equally distanced entrances created around the cup rim to create monitoring tools. A roll of corrugated card (length = 30 cm; width = 3 cm) was inserted into each monitoring tool to provide shelter. Two experimental arenas were prepared depending on the number of vine weevil adults being released (either one or ten). Monitoring tools were positioned 15 cm from one another in a hexagon centrally positioned within a white mesh cage (47.5 × 47.5 × 47.5

cm) for replicates using one vine weevil adult while monitoring tools were positioned 20 cm from one another in a hexagon centrally positioned within a larger white mesh cage (57.5 × 57.5 × 57.5 cm; MegaView Science Co., Ltd., Taichung, Taiwan) for replicates using ten vine weevil adults.



**Figure 3:** Arrangement within each mesh cage for the monitoring tool colour (multiple) experiment.

Each monitoring tool position was randomly altered between replicates to account for potential directional bias. A piece of damp cotton wool and yew leaves were placed in the centre of each cage to provide a source of food and moisture. A total of 50 individuals were tested (five replicates per day for ten days) releasing a single vine weevil adult, while 200 individuals were tested (four replicates per day for five days) for replicates releasing ten vine weevil adults. The timing of weevil release and assessment was the same as previously described.



**Figure 4.** Reflectance values from spectral analysis of coloured paper cups used in trapping experiments and measured using Flame Miniature Spectrometer, Ocean Insight, Duiven, The Netherlands.

### 2.2.3 Monitoring Tool Height

The behavioural response of adult vine weevil to differently sized monitoring tools was tested in a three-choice experiment under the same controlled environment conditions as previously described. Monitoring tools were modified from paper cups (height = 11.3 cm;  $\emptyset$  base = 5.8 cm;  $\emptyset$  rim = 9.8 cm) by removing the cup base and sides to create three heights: 11.3 cm, 6 cm and 3 cm. For the cups to be used as monitoring tools they were inverted so that the rim became the refuge base and four equally distanced entrances created around the rim. As the cups were inverted to create monitoring tools, cup rims were left unaltered, except for inclusion of entrances, to ensure bases were consistent between treatments ( $\emptyset$  rim = 9.8 cm). Monitoring tools were painted externally and internally black using poster paint.

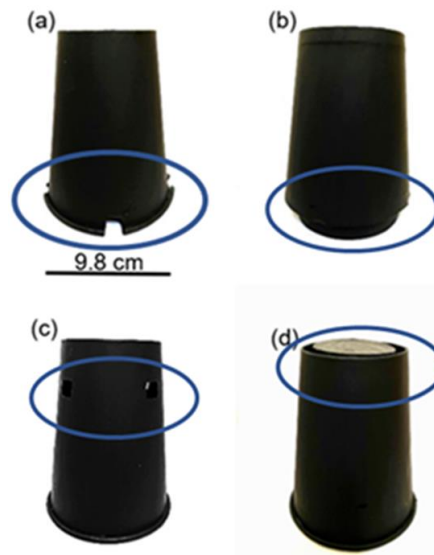
A roll of corrugated card (length = 30 cm; width = 3 cm) was inserted into each refuge to provide shelter as for the previous experiments. Experimental arenas were prepared by placing three monitoring tools (one of each height) in a triangle 30 cm from one another inside a white mesh cage (47.5 × 47.5 × 47.4 cm). Each refuge position was randomly altered between replicates to account for potential directional bias. A piece of damp cotton wool and yew leaves were placed in the centre of each cage to provide a source of food and moisture. Ten adult vine weevils were released from the centre of each cage. A total of 200 individuals were tested (four replicates per day for five days) during this experiment. The timing of weevil release and assessment was the same as previously described.

### 2.2.4 Monitoring Tool Entrance Position

The behavioural response of adult vine weevil to monitoring tools with differing entrance positions was tested in a four-choice experiment under the controlled environment conditions as described previously. All monitoring tools were modified from paper cups (height = 11.3 cm;  $\emptyset$  base = 5.8 cm;  $\emptyset$  rim = 9.8 cm) to create four different entrance configurations: (1) four 1 cm<sup>2</sup> entrances equally distanced from one another around the monitoring tool base, (2) one 1 cm high continuous entrance around the monitoring tool base, (3) four 1 cm<sup>2</sup> entrances equally distanced from one another 8.3 cm from the monitoring tool base and (4) four 1 cm<sup>2</sup> entrances equally distanced from one another at the top of the monitoring tool (Fig. 5).

Monitoring tools were painted externally and internally black using poster paint. Experimental arenas were prepared placing by four monitoring tools (one of each entrance configuration) in the corners of a 30 cm<sup>2</sup> square centrally positioned within a white mesh cage

(47.5 × 47.5 × 47.5 cm). Each refuge position was randomly altered between replicates to account for potential directional bias. A piece of damp cotton wool and yew leaves were placed in the centre of each cage to provide a source of food and moisture. Ten adult vine weevils were released from the centre of each cage. A total of 200 individuals were tested (four replicates per day for five days) during this experiment. The timing of weevil release and assessment was the same as previously described.



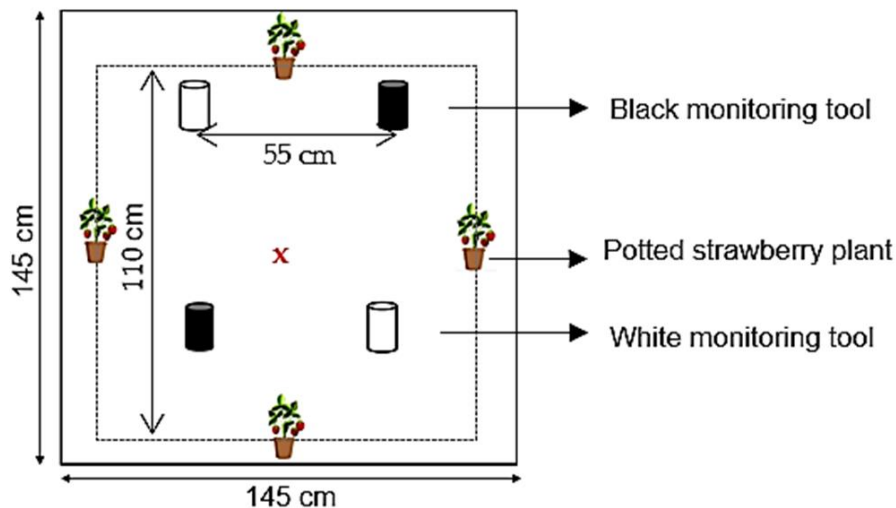
**Figure 5:** Monitoring tools with four different entrance configurations used to test the effect of monitoring tool entrance position on vine weevil adults: (a) four 1 cm<sup>2</sup> entrances equally distanced from one another around the monitoring tool base, b) one 1 cm high continuous entrance around the monitoring tool base, (c) four 1 cm<sup>2</sup> entrances equally distanced from one another 8.3 cm from the monitoring tool base and (d) four 1 cm<sup>2</sup> entrances equally distanced from one another at the top of the monitoring tool.

## 2.3 Glasshouse Experiments

### 2.3.1 Experimental Set-Up

Experiments testing vine weevil behaviour towards different monitoring tool characteristics were carried out in a glasshouse representing a semi-field environment. This glasshouse environment was created using potted (Ø = 13 cm; Teku VCH13, Pöppelmann, Lohne, Germany) strawberry plants (cv. Elsanta; RW Walpole, King's Lynn, UK) located in a fine mesh tent cage (145 × 145 × 152 cm) (Insectopia, Austrey, UK) situated within a glasshouse (mean temperature 19.2 ± 0.8 °C, mean humidity 52.2 ± 1.6 %). Four potted strawberry plants were positioned equidistant from one another along the perimeter of a 110 cm<sup>2</sup> square centrally positioned within the tent cage (Fig. 6), providing both a food source and alternative refuges. Unless otherwise stated monitoring tools were created from paper cups

(height = 11.3 cm,  $\varnothing$  base= 5.8 cm,  $\varnothing$  rim= 8.9 cm) (Comfy Package, New York, US), externally and internally painted black using poster paint (Galeria Acrylic, Windsor & Newton, London, UK). Paper cups were inverted so that the rim became the monitoring tool base and four equally distanced entrances were made in the monitoring tool by cutting 1 cm<sup>2</sup> openings around the cup rim. A roll of corrugated card (length 30 cm, width 3 cm) was inserted into each monitoring tool to provide shelter by exploiting the thigmotactic behaviour exhibited by this species.



**Figure 6.** Schematic diagram showing the arrangement within each tent cage for the monitoring tool colour/ shade experiment. The vine weevil release point is shown by **x**.

### 2.3.2 Monitoring Tool Colour (Light vs Dark)

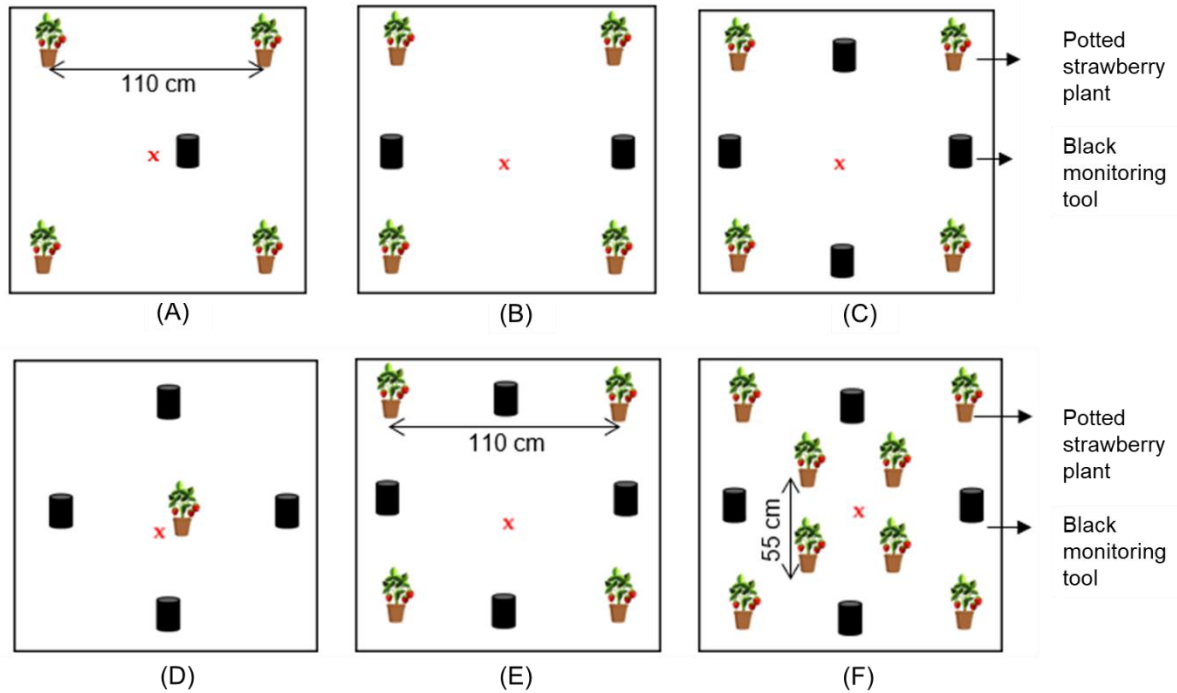
The behavioural response of adult vine weevils to monitoring tool colour was tested in a binary-choice experiment under the semi-field conditions described in Section 2.3.1. Alongside the standard black monitoring tools, white ones were created by painting paper cups white using poster paint (Galeria Acrylic). Experimental arenas were completed by placing four monitoring tools (two black and two white) at the corners of a 55 cm<sup>2</sup> square centrally positioned within the tent cage (Fig. 6). Monitoring tools and the tent cage ( $n = 2$ ), to which monitoring tools were allocated, were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box ( $\varnothing = 12$  cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. A total of 400 individuals were tested (two replicates per day for 5 days) during this experiment.

### **2.3.3 Monitoring Tool Height**

The behavioural response of adult vine weevils to monitoring tool height was tested in a binary-choice experiment under the semi-field conditions previously described. Monitoring tools were created as described in the previous section but modified to two heights: 11.3 and 3 cm. Experimental arenas were completed by placing four monitoring tools (two of each height) at the corner of a 55 cm<sup>2</sup> square centrally positioned within the tent cage. Monitoring tools and the tent cage (n = 2) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box (Ø = 12 cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. A total of 400 individuals were tested (two replicates per day for 5 days) during this experiment.

### **2.3.4 Monitoring Tool Diameter**

The behavioural response of adult vine weevils to monitoring tool diameter was tested in a binary-choice experiment under the semi-field conditions previously described. Monitoring tools were created as previously described and using Kraft bowls (height 6.9 cm, Ø base 16.4 cm, Ø rim 18.4 cm; GoCoPack, UK). To ensure heights were consistently 6.9 cm between treatments, paper cups were modified by removing the base and sides to create monitoring tools with the same height as the Kraft bowls but with different diameters: 8.9 and 18.4 cm. For Kraft bowls, four equally distanced entrances were made in the monitoring tool by cutting 2 cm<sup>2</sup> openings around the Kraft bowl rim. Experimental arenas were completed by placing four monitoring tools (two of each diameter) at the corner of a 55 cm<sup>2</sup> square centrally positioned within the tent cage. Monitoring tools and the tent cage (n = 2) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box (Ø = 12 cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. A total of 400 individuals were tested (two replicates per day for 5 days) during this experiment.



**Figure 7.** Schematic diagram showing the arrangement within each tent cage for the monitoring tool (A, B, C) and plant (D, E, F) density experiment. The vine weevil release point is shown by **x**.

### 2.3.5 Monitoring Tool Density

The behavioural response of adult vine weevils to monitoring tool density was tested in a three-choice experiment under the semi-field conditions previously described. Four potted strawberry plants were positioned at the corners of a 110 cm<sup>2</sup> square centrally positioned within the cage (Fig. 7 A–C), providing both a food source and a range of alternative refuges. Monitoring tools were created as previously described. Experimental arenas were completed by placing different numbers of monitoring tools inside the tent cage: one monitoring tool (~ 0.5 monitoring tool/m<sup>2</sup>) (Fig. 7A) positioned in the middle of a centrally positioned 110 cm<sup>2</sup> square within the tent cage, two monitoring tools (~ 0.95 monitoring tool/m<sup>2</sup>) (Fig. 7B) positioned opposite one another along all four sides of a 110 cm<sup>2</sup> square, and four monitoring tools (~ 1.9 monitoring tool/m<sup>2</sup>) (Fig. 7C) positioned equidistant from one another along the perimeter of a 110 cm<sup>2</sup> square. Monitoring tools and the tent cage ( $n = 3$ ) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box ( $\varnothing = 12$  cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following

day. A total of 1200 individuals were tested (three replicates for 10 days) during this experiment.

### **2.3.6 Effect of Plant Density on Vine Weevil Monitoring Tool Performance**

The behavioural response of adult vine weevils to plant density was tested in a three-choice experiment under the semi-field conditions previously described. One, four or eight potted strawberry plants were positioned within a tent cage (Fig. 7D–F). Monitoring tools were created as previously described. Experimental arenas were completed by placing four monitoring tools equidistant from one another along the perimeter of a 110 cm<sup>2</sup> square within the tent cage (Fig. 7D–F). Different numbers of potted strawberry plants were positioned within the tent cages: one plant (~0.5 plants/m<sup>2</sup>) positioned in the middle of a 110 cm<sup>2</sup> square (Fig. 7D) centrally positioned within the tent cage, four plants (~ 1.9 plants/m<sup>2</sup>) positioned at the corners of a 110 cm<sup>2</sup> square (Fig. 7E), and eight plants (~ 3.8 plants/m<sup>2</sup>) positioned at the corners of two squares (55 cm<sup>2</sup> and 110 cm<sup>2</sup> ; Fig. 7F). Monitoring tools and the tent cage (n = 3) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box (Ø = 12 cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. A total of 1200 individuals were tested (three replicates for 10 days) during this experiment.

## **3. Statistical Analysis**

All statistical analyses were carried out using R (Version 4.2.2) (R Core Team, 2022). For binary-choice experiments the number of individuals within a monitoring tool was analysed using an exact binomial test against the null hypothesis that the number of vine weevils seeking refuge had a 50:50 distribution (Roberts *et al.*, 2019a; 2023). Replicated results from each of the choice experiment tested, prior to carrying out statistical analyses, were pooled. All other experiments (e.g., monitoring tool and plant density experiments, *etc.*) were analysed using generalised linear models (GLMs) fitted with a Poisson or quasi-Poisson probability distributions depending on the presence of overdispersion. This was determined using `qcc.overdispersion.test` function from the `qcc` package (Scrucca, 2004), where a *p*-value greater than 0.05 indicated no overdispersion and justifies the use of Poisson distribution.

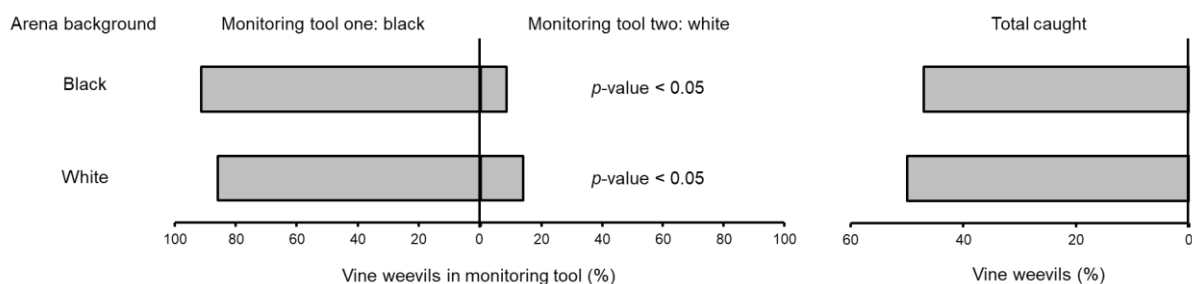
Multiple comparisons for Poisson probability distribution data were evaluated by Tukey's HSD tests implemented in the HSD.test function in the R package agricolae (de Mendiburu, 2019). Individuals not recorded in the monitoring tools were excluded from statistical analysis.

## 4. Results

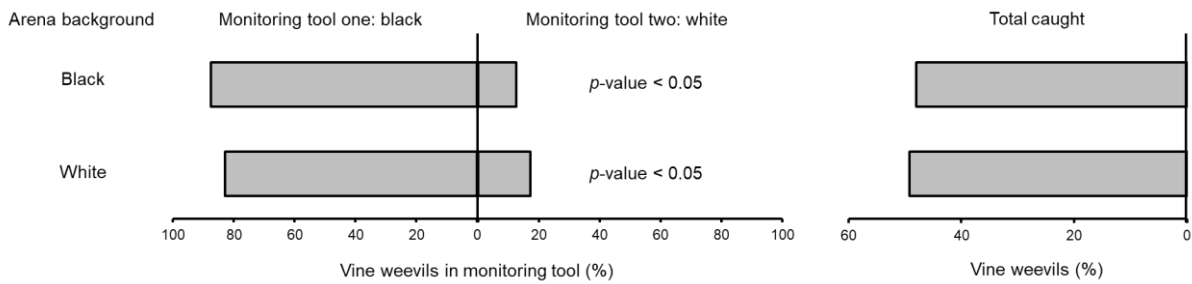
### 4.1 Laboratory Experiments

#### 4.1.1 Monitoring Tool Colour (Dark vs Light)

In binary-choice experiments presenting adult vine weevil with black and white monitoring tools against a black background, the number of individuals recorded in black monitoring tools was significantly higher than in white monitoring tools in both single (Binomial exact test: no. successes = 43, no. trials = 47,  $p < 0.001$ ) (Fig. 8) and group release experiments (Binomial exact test: no. successes = 105, no. trials = 125,  $p < 0.001$ ) (Fig. 9) with 91 % and 88 %, respectively. When the choice between black and white monitoring tools was presented against a white background significantly more vine weevil were recorded in black monitoring tools than white monitoring tools in both individual (Binomial exact test: no. successes = 47, no. trials = 50,  $p < 0.001$ ) (Fig. 8) and group release experiments (Binomial exact test: no. successes = 102, no. trials = 123,  $p < 0.001$ ) (Fig. 9) with 86 % and 83 %, respectively.



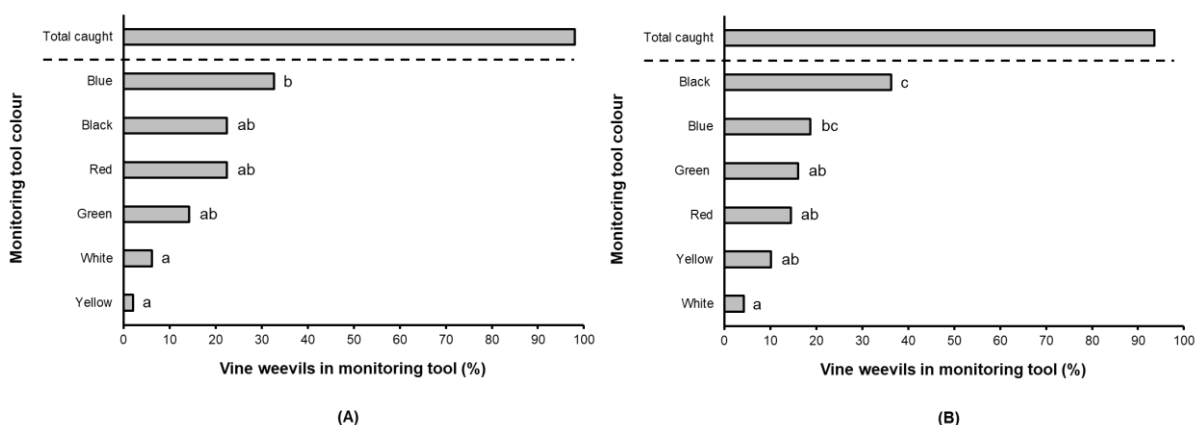
**Figure 8.** Percentage of vine weevil adults recorded in monitoring tools painted black or white when released individually (number of replicate days = 10) into a cage with a black background or white background (Binomial exact test:  $p < 0.05$ ).



**Figure 9.** Percentage of vine weevil adults recorded in monitoring tools painted black or white when released as a group of five individuals (number of replicate days = 5) into a cage with a black background or white background (Binomial exact test:  $p < 0.05$ ).

#### 4.1.2 Monitoring Tool Colour (Multiple)

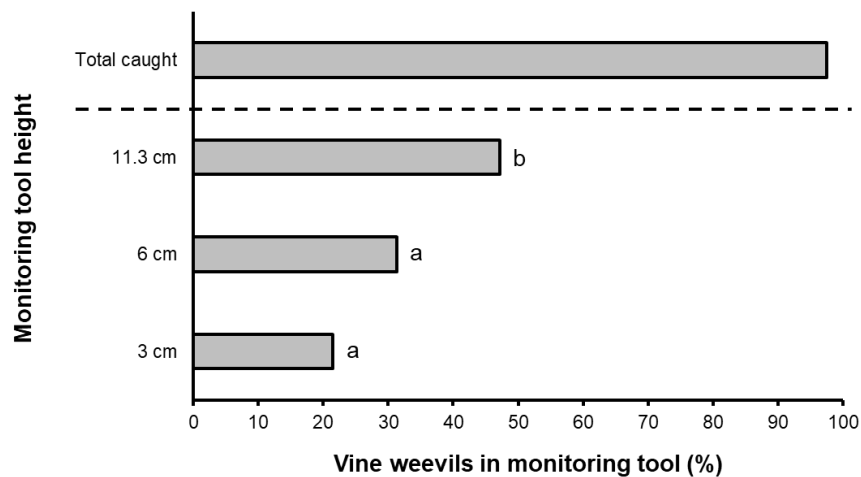
In six-choice experiments presenting adult vine weevil with black, white, blue, green, red and yellow monitoring tools against a white background there was a significant difference in individuals recorded in monitoring tools for both single (Generalised linear model with a quasi-Poisson distribution:  $F^2 = 241.07$ ,  $df = 294$ ,  $p < 0.05$ ) and group release experiments (Generalised linear model with a quasi-Poisson distribution:  $F^2 = 157.47$ ,  $df = 114$ ,  $p < 0.05$ ) (Fig. 10). The percentage of adult vine weevil recorded in blue (32.65 %), black (22.45 %), red (22.45 %) and green (14.28 %) (Fig. 10A) monitoring tools was greater than white (6.12 %) or yellow (2.04 %) monitoring tools when adult vine weevil were released individually (Tukey's HSD test:  $p < 0.05$ ). Black monitoring tools contained the largest number of adult vine weevil when released in groups (36.36 %) (Fig. 10B) though this was not significantly greater than blue (18.72 %) monitoring tools (Tukey's HSD test:  $p > 0.05$ ).



**Figure 10.** Percentage of vine weevil adults recorded in monitoring tools painted blue, green, red, yellow, black or white when released (A) individually (number of replicate days = 10) or (B) as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test:  $p < 0.05$ ).

### 4.1.3 Monitoring Tool Height

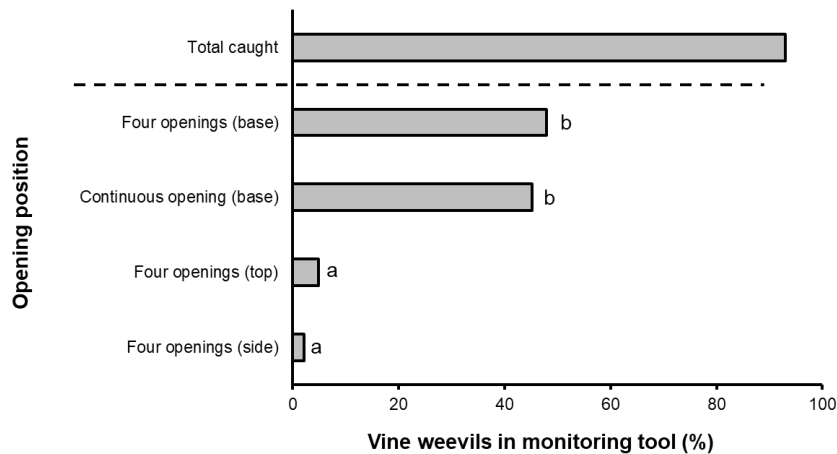
In three-choice experiments presenting adult vine weevil with three monitoring tools of varying heights against a white background, height had a significant effect on the number of weevil adults recorded in the refuge (Generalised linear model with a quasi-Poisson distribution:  $F^2 = 52.09$ ,  $df = 57$ ,  $p < 0.05$ ) (Fig. 11). A higher percentage of adult vine weevils were recorded in the taller monitoring tools (11.30 cm; 47 %) than the medium (6 cm; 31 %) and short (3 cm; 22 %) monitoring tools (Tukey's HSD test:  $p < 0.05$ ).



**Figure 11.** Percentage of vine weevil adults recorded in monitoring tools with a height of 3, 6 or 11.30 cm when released as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test:  $p < 0.05$ ).

### 4.1.4 Monitoring Tool Entrance Positions

In four-choice experiments presenting adult vine weevil with four monitoring tools that had varying entrance configurations against a white background, entrance position had a significant effect on the number of individuals recorded in the monitoring tool (Generalised linear model with a quasi-Poisson distribution:  $F^2 = 62.19$ ,  $df = 76$ ,  $p < 0.05$ ) (Fig. 12). Monitoring tools with entrances positioned around their base contained higher percentages of adult vine weevil (48 % in monitoring tools with four entrances; 45 % in monitoring tools with a continuous entrance) compared to those with entrances at the top (5 %) or in the middle (2 %) (Tukey's HSD test:  $p < 0.05$ ).



**Figure 12.** Percentage of vine weevil adults recorded in monitoring tools with openings on the side, top or base - continuous opening or four openings - when released as a group of ten individuals (number of replicate days = 5). Different letters denote significant differences between means (Tukey's HSD test:  $p < 0.05$ ).

## 4.2 Glasshouse experiments

### 4.2.1 Monitoring Tool Colour (Dark vs. Light)

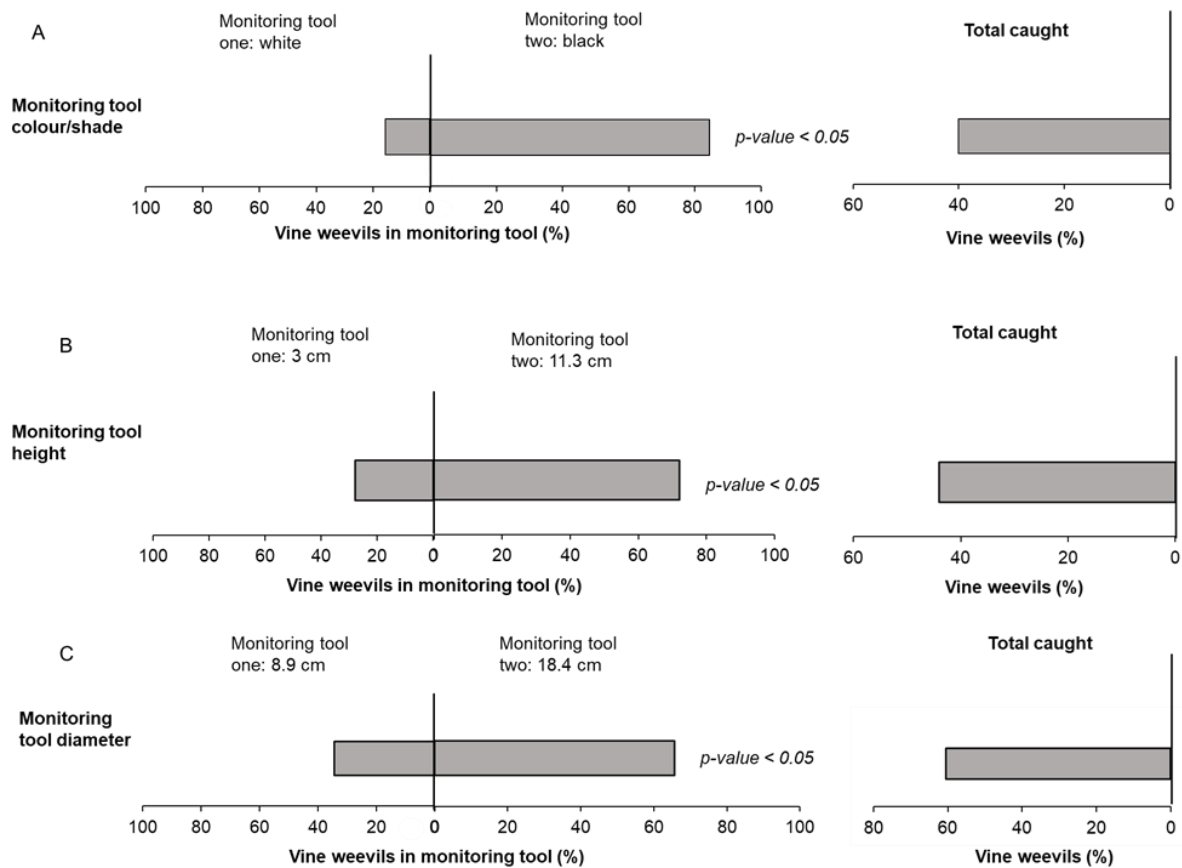
In binary-choice experiments presenting adult vine weevils with black and white monitoring tools, the number of individuals recorded in black monitoring tools was significantly higher than in white monitoring tools (Binomial exact test: no. successes = 135, no. trials = 160,  $p < 0.001$ ) with 84 % and 16 %, respectively. Total catch was 40 % of the introduced weevil population (Fig. 13A).

### 4.2.2 Monitoring Tool Height

In binary-choice experiments presenting adult vine weevils with monitoring tools of varying heights, the number of individuals recorded in taller tools was significantly higher than in shorter monitoring tools (Binomial exact test: no. successes = 127, no. trials = 176,  $p < 0.001$ ) with 72 % and 28 %, respectively. Total catch was 44 % of the introduced weevil population (Fig. 13B).

### 4.2.3 Monitoring Tool Diameter

In binary-choice experiments presenting adult vine weevils with monitoring tools of varying diameters, the number of individuals recorded in large-diameter monitoring tools was significantly higher than smaller-diameter monitoring tools (Binomial exact test: no. successes = 159, no. trials = 242,  $p < 0.001$ ) with 65.7 % and 34.3 %, respectively. Total catch was 60.5 % of the introduced weevil population (Fig. 13C).



**Figure 13.** Percentage of vine weevil adults recorded in monitoring tools when testing (A) monitoring tool colour/shade, (B) monitoring tool height and (C) monitoring tool diameter. Vine weevil adults were released as a group of 40 individuals (number of replicate days = 5) (Binomial exact test:  $p < 0.05$ ).

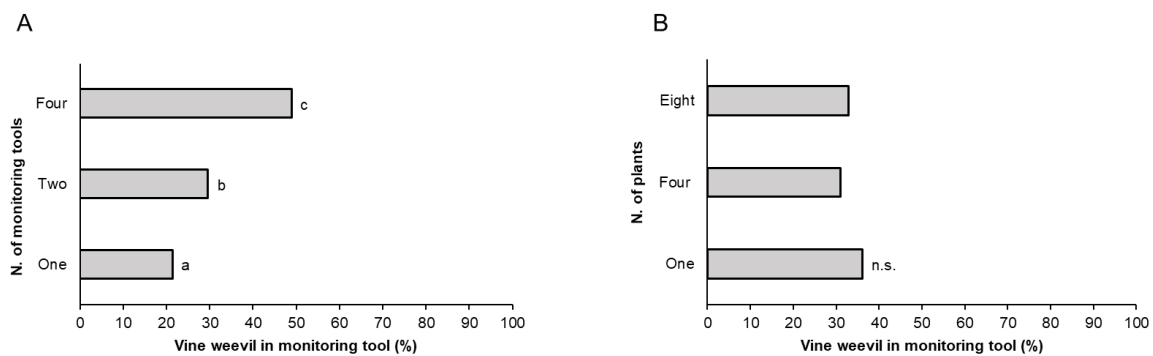
### 4.2.4 Monitoring Tool Density

Monitoring tool density significantly influenced the total number of individuals recorded in the monitoring tools (generalised linear model with a quasi-Poisson distribution:  $F^2 = 121.15$ ,

df = 27,  $p < 0.001$ ). Experimental arenas with four monitoring tools had the highest vine weevil retention (49%) (Tukey's HSD test:  $p < 0.05$ ) while in arenas with two or one monitoring tools the retention rate was only 30 % and 21 % of the introduced vine weevils, respectively (Fig. 14A).

#### 4.2.5 Effect of Plant Density on Vine Weevil Monitoring Tool Performance

Plant density did not significantly influence the number of individuals recorded in monitoring tools (generalised linear model with a quasi-Poisson distribution:  $F^2 = 143.53$ , df = 117,  $p = 0.1$ ). Monitoring tools in experimental arenas containing one plant retained 36.1 % of the introduced vine weevils while in arenas with four plants the retention rate was 31.1 % and in arenas with eight plants it was 32.8 % (Fig. 14B).



**Figure 14.** Percentage of vine weevil adults recorded in monitoring tools when testing (A) monitoring tool density and (B) plant density. Vine weevil adults were released as a group of 40 individuals (number of replicate days = 10). Different letters indicate significant differences between tested monitoring tools (Tukey's HSD test:  $p < 0.05$ ); n.s.= not significant.

## 5 Discussion

Visual cues play an important role in host plant location for insect pests (Prokopy and Owens, 1983). Such cues have been shown to be key components to consider when developing monitoring tools for curculionid species (Bjorklund *et al.*, 2005). Monitoring tool design (Hallett *et al.*, 1999), colour (Silva *et al.*, 2018), density (Faleiro *et al.*, 2011) and position (Reddy *et al.*, 2011) may be used to augment monitoring tool efficacy. Hence, an understanding as to how these factors influence monitoring tool-insect interactions can inform their development for improved efficacy and reliability. Results presented here indicate that adult vine weevil prefer dark monitoring tools (black) over light monitoring tools (white) in two-

choice experiments (Fig. 15) under laboratory conditions. In particular, the number of individuals recorded in black monitoring tools was significantly higher (86 %) than in white monitoring tools (14 %) in individual release experiments. Similarly, the number of individuals recorded in black monitoring tools was significantly higher (83 %) than in white monitoring tools (17 %) in group release experiments. Whereas, in six-choice experiments presenting vine weevil with black, white, blue, green, red and yellow monitoring tools more adult vine weevils were recorded in blue (32.65%), black (22.45%), red (22.45%) and green (14.28%) monitoring tools than white (6.12%) or yellow (2.04%) monitoring tools when adult vine weevil were released individually. Black monitoring tools contained the largest number of adult vine weevil when released in groups (36.36%). To further explore monitoring tool colour preference and confirm whether black monitoring tools may be used as an effective method to detect this species, this study investigated the efficacy of black monitoring tools also under glasshouse conditions. The results confirmed that black monitoring tools are more effective than white ones under field representative environmental conditions. In fact, the number of individuals recorded in black monitoring tools was significantly higher (84 %) than in white monitoring tools (16 %).

These findings suggest that vine weevil show a preference for certain colours, particularly dark ones. However, further research is needed to investigate the visual capabilities of vine weevils, including their sensitivity to a wider range of colours (e.g., similar to studies with the red palm weevil; Reddy *et al.*, 2011). Studies exploring how vine weevils perceive and respond to different colours could provide deeper insights into their colour preferences and improve vine weevil monitoring tool design. In addition, although reflectance values of the coloured paper cups used in these experiments have been provided (Figure 4), it would be particularly interesting to investigate whether vine weevils are sensitive to a broader range of light wavelengths, including ultraviolet (UV) and red (e.g., Ilić *et al.*, 2016). Understanding whether vine weevils can perceive colours beyond the range of human vision could provide valuable insights into their visual preferences and potentially inform the development of even more effective monitoring tools.

Monitoring tool colour is a key factor that influences monitoring tool-insect interactions (Mahot *et al.*, 2020). While no comparable studies have been carried out with vine weevils, these results support studies carried out with the red palm weevils (*Rhynchophorus ferrugineus* Olivier) where black inverted bucket monitoring tools caught more individuals than white ones (Hallet *et al.*, 1999). The importance of using dark colours to increase monitoring tool efficacy for weevils has been previously reported (Abuagla and Al-Deeb, 2012). For example, the New Guinea sugarcane weevil (*Rhabdoscelus obscurus* Boisduval) shows a preference for russet-brown colours in field conditions (Reddy *et al.*, 2011), whereas the apple

blossom weevil (*Anthonomus pomorum* Linnaeus) exhibits a preference for blue coloured monitoring tools (Hausmann *et al.*, 2004).

Differences in luminance or coloration due to surface patterns or shadows may be perceived as contrast signals. Insects use contrast to discriminate objects from their background and to recognise shapes (Prokopy and Owens, 1983; Lehrer and Campan, 2004). Entwistle (1963) and Timmons and Potter (1981) speculated that insects caught by red, brown and black traps are likely responding to dark shades and the contrast with the background rather than to any visual cue. This study contradicts this hypothesis as differences between background colour, and therefore monitoring tool contrast, did not influence adult vine weevil behaviour. Adult vine weevils, whether released individually or in groups, preferred black monitoring tools over white ones in two-choice experiments, regardless of whether the background was white or black. However, this result will require further investigation to fully understand how vine weevil adults perceive objects in different environments.

There are a range of other factors that may influence monitoring tool efficacy. Silva *et al.* (2018) showed that boll weevil traps, designed to mimic the colour of foliage, were less effective when used for cranberry weevil (*Anthonomus musculus* Say) adults than sticky traps of similar colour. This suggests that trap shape, and not only colour, is important for cranberry weevil. Shape is known to be a key factor influencing insect monitoring tool efficacy (Reddy *et al.*, 2005, 2009; Sajeewani *et al.*, 2020). Monitoring tool height impacts the shape and has been shown to directly influence the behaviour of target organisms. Tree-of-heaven root weevil (*Eucryptorrhynchus scrobiculatus* Motschulsky) and the walking white pine weevil (*Pissodes strobi* Peck) adults orientated preferentially towards silhouettes that are taller rather than shorter (Vandersar and Borden, 1977; Wang *et al.*, 2019). This is in accordance with this study where the number of vine weevils retained within the monitoring tools was higher in taller monitoring tools (72 %) than in shorter monitoring tools (28 %), suggesting that taller silhouettes may be an important cue also for this weevil. Similarly, the number of vine weevil adults was higher in larger-diameter monitoring tools (65.7 %) than monitoring tools with a smaller diameter (34.3 %)(Fig. 15). Monitoring tool diameter has been reported to significantly affect trapping efficiency in some species of moth (Yongmo *et al.*, 2005; Zhao *et al.*, 2012) and typically the efficacy of a monitoring tool increases with the size of the tool used (Reddy *et al.*, 2009).

Roberts *et al.* (2019a) found that despite the general design (colour and silhouette) of vine weevil and red palm weevil traps being similar, their efficacy differed due to other characteristics including the entrance position. The results demonstrated that monitoring tools with openings at their base had higher percentages of vine weevil adults (48 % in monitoring tools with four entrances; 45 % in monitoring tools with a continuous entrance) compared to those with entrances at the top (5 %) or in the middle (2 %) (Fig. 15). Although many studies

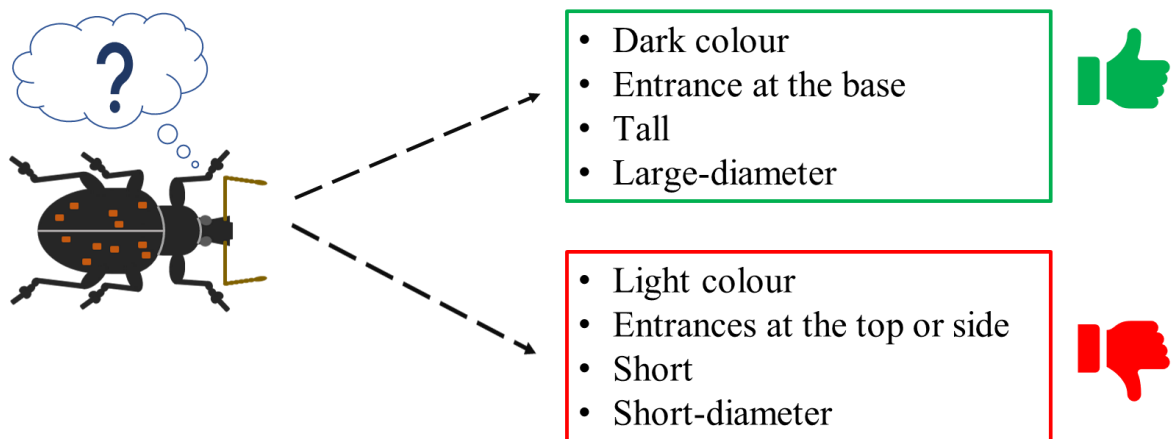
have assessed the effect of monitoring tool design on efficacy, there are, to date, no studies that have specifically examined the effect of entrance position and have instead focused on the effect of entrance number and spacing (Epsky and Shuman, 2002; Toews and Phillips, 2002; Lee *et al.*, 2012).

Other visual characteristics, including design, shape, texture and location, may all affect monitoring tool efficacy. For example, Reddy *et al.* (2012) showed that Pherocon unitraps caught higher numbers of the sweet potato weevil (*Cylas formicarius* Fabricius; Coleoptera: Brentidae), than ground, funnel water or delta traps. Previous studies have also reported that the responses of insect pests to the visual characteristics (e.g., colour, height, design) of monitoring tools may be affected by environmental factors, including soil type, temperature, humidity and light intensity (Tinzaara *et al.*, 2005), which may vary through the day and the seasons. Increasing knowledge on how vine weevil adults respond to environmental change may provide insights into the development of a more effective monitoring tool for this species.

This study demonstrates that monitoring tool density positively correlates with the proportion of the vine weevil population recorded within monitoring tools. Experimental arenas with four monitoring tools recorded the highest percentage of vine weevil adults (49 %) compared those with two (30 %) or one monitoring tool (21 %). These results are in line with previous studies showing that monitoring efficacy can be improved by increasing the density of monitoring tools (Tinzaara *et al.*, 2005; Faleiro *et al.*, 2011). Vidyasagar *et al.* (2016) showed that a mean of 61.5 red palm weevils (*R. ferrugineus* Olivier) were captured when eight monitoring tools were deployed for every 4 ha compared to a mean of 10.0 weevils captured when one monitoring tool was deployed for every 4 ha. In addition to monitoring tool density, it is necessary to consider the number of plants in an area (Spear-O'mara and Allen, 2007) as well as the area covered (Faleiro *et al.*, 2011). In this study the effect of plant density on monitoring tool efficacy was evaluated. The results showed that the number of plants within a constant area and with a known vine weevil population did not influence the number of weevils recorded within monitoring tools. In a previous study the number of saddled prominent moths (*Heterocampa guttivitta* Walker, Lepidoptera: Notodontidae) recorded in monitoring tools increased when the abundance and density of the host plant (sugar maple, *Acer saccharum* Marshall) also increased (Spear-O'mara and Allen, 2007). By contrast, Westerberg *et al.* (2021) showed that the number of pollinating insects in pan traps decreased as the abundance of surrounding flowers increased. This pattern may then differ between taxa and depend on flower colours, spatial scale and timing of the monitoring activity.

This study investigated factors that may enhance monitoring tool efficacy by comparing the effects of visual cues on vine weevil retention. Based on the experimental results generated in this study, the most effective monitoring tool design is dark (black), tall with a

large diameter and with entrances on the base (Fig. 15). By increasing the number of monitoring tools per unit area, the proportion of vine weevil population recorded could be increased. However, further investigations are needed in order to provide a better understanding of vine weevil visual ecology and how they interact with monitoring tools will help inform their optimisation and improve detection and monitoring of this pest.



**Figure 15:** Schematic diagram showing a summary of vine weevil adult preferences to some monitoring tools visual characteristics (colour, height, diameter and entrance positions).

# Chapter 3: Effect of Photoperiod and Olfactory Cues on Vine Weevil Behaviour and Monitoring Tool Efficacy<sup>2</sup>

## Abstract

Vine weevil chemical ecology has been extensively investigated in efforts to create a semiochemical lure that improves the accuracy and sensitivity of monitoring tools, but little is known about the effects of light and photoperiod on life history traits of vine weevil and whether they modulate vine weevil responses to olfactory cues. This study investigated the behavioural responses of adult vine weevils to olfactory (host plant and conspecifics) cues under different light conditions (day, dawn and night) and photoperiods (normal and reversed) using Y-tube olfactometer bioassays. In addition, to improve vine weevil monitoring the behavioural responses of adult vine weevils to olfactory (host plant and conspecifics) cues are investigated under glasshouse conditions. Results showed that, in laboratory bioassays, under 'day conditions' vine weevil adults preferred odours derived from host plant (yew) foliage or conspecifics whereas under dawn and night conditions no preferences were recorded between stimuli. Similarly, no difference in the choices of adults were recorded when vine weevil adults were presented with odours at different points in their circadian rhythm (e.g., normal or reversed photoperiod). Under glasshouse conditions, more vine weevils were recorded in monitoring tools baited with yew and conspecifics than in unbaited monitoring tools or those baited with only yew. Baiting monitoring tools with conspecifics alone did not enhance the number of vine weevils recorded in monitoring tools.

This study provides information on key olfactory factors that influence vine weevil behaviour. Understanding how this species modulates olfactory responses induced by environment changes, including light conditions may help to improve vine weevil monitoring strategies.

## 1. Introduction

Semiochemicals are chemical compound (both individual or mixture) released from one organism that evoke either a behavioural or physiological response in another organism

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<sup>2</sup> Sections of the following Chapter have been published in:

Fezza *et al.* (2023). Decoding attraction: Improving vine weevil monitoring by exploiting key sensory cues. *Pest Management Science*, 79 (11), pp. 4635-4643. <https://doi.org/10.1002/ps.7665>.

(Abd El-Ghany, 2019). Volatile organic compounds (VOCs) isolated from either host plants or conspecifics are often used to manipulate insect behaviour and enhance monitoring tool sensitivity by increasing catch rates (Cano-Ramírez *et al.*, 2012; García-Díaz *et al.*, 2020) even at low densities (Mahroof and Phillips, 2008).

The addition of semiochemicals, such as pheromones and/or host plant volatiles, have been shown to improve the efficacy of monitoring tools for Curculionidae pest species (Oehlschlager *et al.*, 2002; Reddy *et al.*, 2009). For example, ramp traps baited with the aggregation pheromone sordidin (Cosmolure+) caught significantly ( $11.1 \pm 0.4$  adults per trap) more banana root borer (*Cosmopolites sordidus* German; Coleoptera: Curculionidae) adults than those without pheromone lures ( $0.6 \pm 0.1$  adults per trap) (Reddy *et al.*, 2008). Similarly, monitoring tools baited with host plant volatiles (e.g.,  $\alpha$ -pinene and ethanol) caught more pine bark beetles (*Hylastes ater* Paykull; Coleoptera: Curculionidae) and red-haired pine bark beetles (*Hylurgus ligniperda* Fabricius; Curculionidae: Scolytinae) than unbaited traps (Kerr *et al.*, 2017). Pheromones associated with the pest species may also be used together with host plant volatiles to enhance monitoring sensitivity and reliability. For example, Evenden *et al.* (2016) showed that the addition of host plant volatiles from faba beans (*Vicia faba* L.; Fabales: Fabaceae) to an aggregation pheromone lure enhanced lure effectiveness for trapping the pea leaf weevil (*Sitona lineatus* L.; Coleoptera: Curculionidae). The use of semiochemicals, such as pheromones, that could be used as a lure to enhance vine weevil monitoring tool efficacy is limited by the fact that adults, which reproduce parthenogenetically, are not thought to produce a sex pheromone (Pope and Roberts, 2022).

Vine weevil adults do, however, display a strong aggregation behaviour (Kakizaki, 2001) and adults prefer refuges previously occupied by their conspecifics (Pickett *et al.*, 1996). It is not known what mediates this aggregation behaviour, but it is hypothesised to be moderated by a pheromone, though this has proved challenging to identify (Pickett *et al.*, 1996). For this reason, most work investigating vine weevil lures has focused on identifying volatiles produced by their host plants (Pope and Roberts, 2022). Behavioural studies have shown that adult vine weevils respond to a wide range of plant odours (van Tol and Visser, 1998, 2002; van Tol *et al.*, 2002, 2004; Roberts *et al.*, 2019 a, b). However, to date, no effective and reliable synthetic lure has been identified for use in a monitoring tool system (Pope and Roberts, 2022).

Insect responses to biologically active chemical stimuli vary according to biotic and abiotic environmental factors (Gadenne *et al.*, 2016). Circadian rhythms of sensitivity and orientation towards olfactory cues (e.g., host plant and pheromone) have been reported in the tsetse flies (e.g., *Glossina morsitans morsitans*; Diptera: Glossinidae) as well as in some moths (e.g., *Trichoplusia ni*; Lepidoptera: Noctuidae) and cockroaches (e.g., *Supella longipalpa*; Blattodea: Ectobiidae) (Liang and Schal, 1990; Linn *et al.*, 1996; Van Der Goes

Van Naters *et al.*, 1998). The 'kissing bug' (*Triatoma infestans* Klug; Hemiptera: Reduviidae), the most important vector of the Chagas disease, has been shown a daily rhythm of orientation towards the food source (Barrozo *et al.*, 2004). In particular, when larvae were tested under three different photoperiods (e.g., constant darkness, constant light and 12:12 hours light:dark cycles), they showed a positive orientation response towards carbon dioxide during the first hours of the scotophase only (Barrozo *et al.*, 2004). Similarly, in another study, fruit flies (*Drosophila melanogaster* Meigen; Diptera: Drosophilidae) were exposed to a series of olfactory stimuli and their behavioural responses were assessed by measuring the amplitude of electroantennograms (EAGs) (Krishnan *et al.*, 1999). When the mean EAG amplitudes recorded at different times of day were compared, flies tested around the middle of the night exhibited significantly higher responses than those tested at other times, even when kept in constant darkness. These observations indicate that circadian rhythms may play a broader role in shaping olfactory behaviour in insects, prompting further questions about where rhythmicity is imposed within the olfactory pathway.

Light duration (i.e., photoperiod) and intensity are also key environmental factors affecting insect ecology, including feeding rate, fecundity, survival and development rate (Saunders, 1981; Gómez-Vidal *et al.*, 2006; Zhang *et al.*, 2010). For example, the pine root collar weevil (*Hylobius radicis* Buchanan; Coleoptera: Curculionidae) adult behaviour and movement have shown to be influenced by light intensity. During the day this weevil remains in the vicinity of the root collar of young red Pine (*Pinus resinosa* Sol. ex Aiton; Pinaceae) plantations where their activities include feeding on the bark of the bottom branches, mating and ovipositing but move away from it as light intensity reaches a very low level near the end of the twilight period (Wilson, 1968). After that, they return to the root collar again once light intensity increases at dawn (Wilson, 1968). In vine weevil, the adults spend the night close the host plant where their activities include feeding on leaves and ovipositing, however as dawn approaches, they look for a refuge where they tend to aggregate (Smith, 1932). Therefore, it is suggested that environmental factors such as light intensity and duration may influence host plant-vine weevil interactions as well as conspecific interactions.

The aim of this study was to evaluate whether: (i) there is variation among behavioural responses to olfactory stimuli (e.g., host plant and conspecifics) under different light conditions; (ii) vine weevil adults show a preference for volatiles released from host plant material and/or conspecific baited monitoring tools. It is hypothesised that: (i) light conditions and photoperiods influence vine weevil preference for host plant and conspecifics olfactory stimuli under laboratory conditions, and (ii) adult vine weevil responses to monitoring tools under glasshouse conditions is influenced by the presence of volatiles associated with host plant material and/or conspecifics.

## 2. Materials and Methods

### 2.1 Insects

Adult vine weevils were collected in June 2021 and July- August 2022 from commercial strawberry (*Fragaria x ananassa*; Rosales: Rosaceae) crops grown in Staffordshire (UK). These individuals were maintained on yew (*Taxus baccata* L.; Cupressales: Taxaceae) sprigs and moist paper towels that were replaced weekly in plastic terrariums (30 × 19.3 × 20.6 cm; Exo Terra, Castleford, UK). Thirty-five to forty weevils were housed within each terrarium and maintained in a controlled environment room (20 °C, 60% relative humidity, 16:8 hours light:dark photoperiod; Fitotron, Weiss Technik, Ebbw Vale, Wales, UK) until their use in experiments. The first batch of vine weevil adults (collected in June 2021) was used for Y-tube olfactometer bioassays carried out between February and May 2022 under laboratory conditions, while the second batch (collected in July- August 2022) was used for the experiments carried out under glasshouse conditions (October-November 2022).

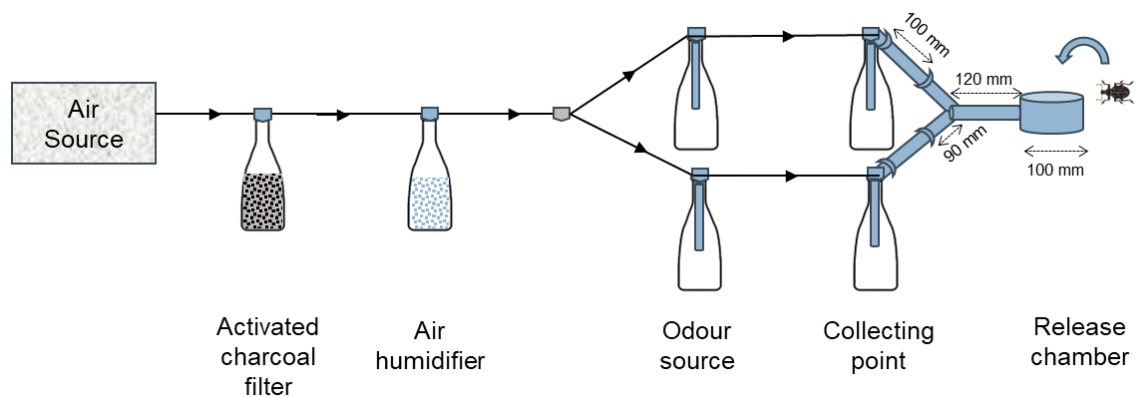
### 2.2 Laboratory Experiments

#### 2.2.1 Y-tube Olfactometer Bioassay

The behavioural responses of adult vine weevils to chemical stimuli were tested using a glass Y-tube olfactometer (Sci-Glass Consultancy, Bere Alston, UK) (Roberts *et al.*, 2023) following the design previously used by Roberts *et al.* (2019b) (Fig. 1). The olfactometer consisted of a 120 mm long glass tube that branched into two 190 mm arms with an internal diameter of 18 mm. A weevil release chamber ( $\varnothing = 100$  mm) was connected to the base of the Y-tube olfactometer. The airflow was purified by passing it through a charcoal filter, an air humidifier (tap water) and then passed through two serially connected Drechsel bottles (500 ml) and connecting arms before entering the Y-tube. The airflow was set to 1200 ml.min<sup>-1</sup> with odour sources held in the first set of Drechsel bottles for at least one hour before an experiment began. An empty Dreschel bottle was used for the control. All bioassays were undertaken in a controlled environment room (20 °C, 60 % relative humidity; Fitotron, Weiss Technik, Ebbw Vale, Wales, UK) between 07:00 and 11:00 am.

The olfactory stimuli used in the Y-tube bioassays comprise twenty adult vine weevils or small branches of yew (*T. baccata*) (20 g,  $\varnothing \sim 1$  cm and length  $\sim 10$  cm) previously identified as eliciting a response to vine weevil adults (van Tol *et al.*, 2002; 2004; Roberts *et al.*, 2019 a, b). Freshly cut branches of yew were collected from Harper Adams University grounds and used in experiments within four hours.

Prior to use in a bioassay, vine weevil adults were starved for forty-eight hours. A single vine weevil (approximately ten-month-old) was introduced into the olfactometer via a release chamber (Fig. 1) using a fine brush. A choice was recorded when the weevil reached the collecting point (Fig. 1) at the end of the branched arms. Non-responding adults were recorded as such. Each pair of odour sources (e.g., yew or conspecifics vs. control) was tested thirty times using fresh individuals and the numbers of weevils that reached the end of each arm within the 20-minutes testing period was recorded. To minimize any potential directional bias, the position of the odour sources was alternated between replicates. After each test, all glassware was thoroughly cleaned by rinsing with warm water followed by HPLC-grade acetone (Sigma Aldrich; Dorset, UK) and then baked in a glassware oven at 120 °C for 15 minutes (Roberts *et al.*, 2019 b).



**Figure 1.** Schematic diagram of the Y-tube olfactometer used for two-choice preference tests. Each arm of the olfactometer was connected to an odour source contained in the Drechsel bottle. Air (1200 ml.min<sup>-1</sup>) pumped through a charcoal filter was humidified and blown into each odour Drechsel bottle.

### 2.2.2 Effect of Light

The behavioural response of adult vine weevils to different light conditions was tested in a Y-tube bioassay investigating individual responses to olfactory cues using the olfactometer setup previously described (Fig. 1). Fluorescent tubes (Philips Master TL-D 70W/840, UK) were set up to produce three different light conditions: total light (simulating 'day light'; 708 Lux), partial light (simulating 'dawn light'; 138 Lux) and total darkness (simulating 'night'; 0 Lux) within a controlled environment (20 °C; 60% relative humidity; Fitotron). A single weevil was introduced in the olfactometer via a release chamber using a fine brush. Each bioassay replicate time lasted twenty minutes with a maximum of six individuals tested per day (thirty individuals per each binary choice experiment). To avoid the

influence from learned experience (Roberts *et al.*, 2019a), each weevil was used only once. All bioassays were undertaken between 07:00 and 11:00 am.

### **2.2.3 Effect of Light and Photoperiod**

The behavioural response of adult vine weevils to different light conditions and photoperiods was tested in a Y-tube bioassay investigating individual responses to olfactory cues using the olfactometer setup previously described (Fig. 1). Fluorescent tubes (Philips Master TL-D 70W/840, UK) were set up to produce three different light conditions within a controlled environment (20 °C; 60% relative humidity; Fitotron) as previously described (Section 2.1.2). In addition, to investigate the effect of photoperiod, two photoperiods were used: a standard (16:8 hours light:dark; scotophase from 22:00 to 6:00) photoperiod or reversed photoperiod with the scotophase from 06:00 to 14:00 hours. Prior to the experiment, vine weevil adults were conditioned to their assigned photoperiod for ten days in a controlled cabinet (Panasonic, MLR- 352- PE, Japan) set to 20 °C. At the end of the condition period a single weevil was introduced in the olfactometer via a release chamber using a fine brush. Each bioassay replicate lasted twenty minutes with a maximum of six individuals tested per day (thirty individuals each binary choice experiment). To avoid the influence from learned experience (Roberts *et al.*, 2019a), each weevil was used only once. All bioassays were undertaken between 07:00 and 11:00 am.

## **2.3 Glasshouse Experiments**

### **2.3.1 Experimental Set-up**

Experiments testing vine weevil behaviour towards monitoring tool with or without olfactory cues were carried out in a glasshouse representing a semi-field environment. This glasshouse environment was created using potted ( $\varnothing = 13$  cm; Teku VCH13, Pöppelmann, Lohne, Germany) strawberry plants (cv. *Elsanta*; RW Walpole, King's Lynn, UK) placed in a fine mesh tent cage (145 × 145 × 152 cm) (Insectopia, Austrey, UK) situated within a glasshouse (mean temperature  $19.2 \pm 0.8$  °C, mean humidity  $52.2 \pm 1.6\%$ ). Four potted strawberry plants were positioned equidistant from one another along the perimeter of a 110 cm<sup>2</sup> square centrally positioned within the tent cage, providing both a food source and alternative refuges. Monitoring tools were created from paper cups (height = 11.3 cm,  $\varnothing$  = base 5.8 cm,  $\varnothing$  rim = 8.9 cm) (Comfy Package, New York, US), externally and internally painted black using poster paint (Galeria Acrylic, Windsor & Newton, London, UK). Paper cups

were inverted so that the rim became the monitoring tool base and four equally distanced entrances were made in the monitoring tool by cutting 1 cm<sup>2</sup> openings around the cup rim. Experimental arenas were completed by placing four monitoring tools at the corner of a 55 cm<sup>2</sup> square centrally positioned within the tent cage. A roll of corrugated card (length 30 cm, width 3 cm) was inserted into each refuge to provide shelter by exploiting the thigmotactic behaviour exhibited by this species.

### 2.3.2 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance

The behavioural response of adult vine weevils to olfactory cues was tested in four binary-choice experiments under the semi-field conditions as previously described in Section 2.2.1. Small branches of yew (*T. baccata*) ( $\varnothing$  ~1 cm and length ~4 cm) and/or 20 adult vine weevils were placed in a white organza bag (7 × 9 cm; OWill, UK), which was then placed inside a monitoring tool. For experiment 1 (Table 1) two monitoring tools were baited with 3 g of yew and two contained an empty organza bag. For experiment 2 (Tab. 1) two monitoring tools were baited with 20 weevils and two contained an empty organza bag. For experiment 3 (Table 1) two monitoring tools were baited with 3 g of yew plus 20 weevils and two contained an empty organza bag. In experiment 4 (Table 1) two monitoring tools were baited with 3 g of yew plus 20 weevils and two monitoring tools were baited with 3 g of yew. Monitoring tools and the tent cage (n = 2) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools and odour sources, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box ( $\varnothing$  = 12 cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. For each binary choice experiment (Table 1), a total of 400 individuals were tested (two replicates per day for 5 days).

**Table 1.** Experiments testing the effect of olfactory cues on vine weevil monitoring tool performance

Experiment	Monitoring tool 1	Monitoring tool 2	No. of replicates
1	Unbaited	Yew <sup>a</sup>	5
2	Unbaited	Conspecific <sup>b</sup>	5
3	Unbaited	Yew + conspecific	5
4	Yew	Yew + conspecific	5

<sup>a</sup> Small branches of yew (*Taxus baccata*) ( $\varnothing$  ≈ 1 cm and length ≈ 4 cm) were used for the experiments with yew.

<sup>b</sup> Twenty vine weevil adults were used for the experiments with conspecifics.

### **3. Statistical Analyses**

All statistical analyses were carried out using R (Version 4.2.2) (R Core Team, 2022). Y-tube olfactometer bioassays data were analysed using an exact binomial test against the null hypothesis that the number of vine weevils reaching the end of olfactometer arm had a 50:50 distribution (Roberts *et al.*, 2019b; 2023). Similarly, for binary-choice experiments the number of individuals within a monitoring tool (i.e., monitoring tool performance) was analysed using an exact binomial test against the null hypothesis that the number of vine weevils seeking refuge had a 50:50 distribution.

## **4. Results**

### **4.1 Laboratory Experiments**

#### **4.1.1 Effect of Light**

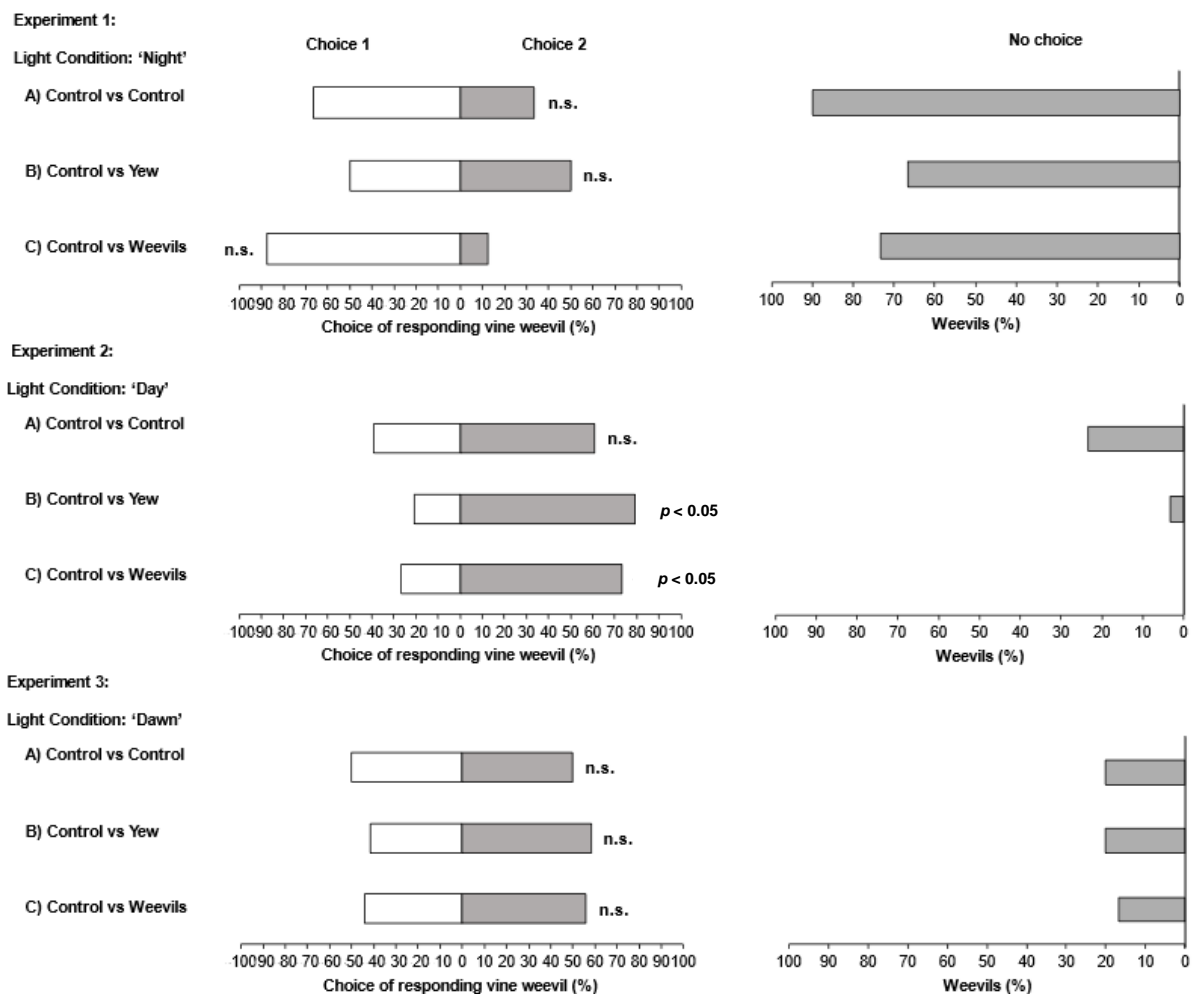
##### **4.1.1.1 'Night'**

In the binary-choice experiments presenting adult vine weevils with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer arm with yew or weevil odours) under 'Night' conditions (Fig. 2, Experiment 1), the number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 5, no. trials = 10,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 1, no. trials = 8,  $p > 0.05$ ) (Fig. 2, Experiment 1). The number of weevils not responding to the choice between control and yew odour was 66.67 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 73.33 % of the introduced weevil population (Fig. 2, Experiment 1).

##### **4.1.1.2 'Day Light'**

In the binary-choice experiments presenting adult vine weevils with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer arm with yew or weevil odours) under a 'Day' condition (Fig. 2, Experiment 2), the number of weevils recorded in the treatment arms with yew was significantly different compared to that in the control arms (binomial test: no. of successes = 23, no. of trials = 29,  $p < 0.05$ ) (Fig. 2, Experiment 2). Similarly, the number of weevils recorded in the treatment arms with weevils was significantly

different compared to that in the control arms (binomial test: no. of successes = 22, no. of trials = 30,  $p > 0.05$ ) (Fig. 2, Experiment 2). In particular, the vine weevils preferred the Y-tube olfactometer arms containing the treatment with yew (79.31 %) and weevils (73.33 %) compared to the control arms, 20.69 % and 26.67 %, respectively. The number of weevils not responding to the choice between control and yew odour was 3.33 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 0 % of the introduced weevil population (Fig. 2, Experiment 2).



**Figure 2:** Behavioural responses of vine weevil adults (%) toward three experimental treatments: (A) control and control, (B) control and Yew, and (C) control and weevils. Each experimental treatment was tested under three different light conditions: Night (Experiment 1), Day (Experiment 2) and Dawn (Experiment 3). For each experimental treatment a single vine weevil adult (number of replicates = 30) was released in the release chamber of the Y- tube olfactometer (binomial exact test:  $p < 0.05$ ); n.s. = not significant.

#### **4.1.1.3 'Dawn Light'**

In the binary-choice experiments presenting adult vine weevils with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer arm with yew or weevil odours) under a 'Dawn' condition (Fig. 2, Experiment 3), the number of weevils recorded in the treatment arms (yew or weevils) was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 14, no. trials = 24,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 14, no. trials = 25,  $p > 0.05$ ) (Fig. 2, Experiment 3). The number of weevils not responding to the choice between control and yew odour was 20 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 16.67 % of the introduced weevil population (Fig. 2, Experiment 3).

#### **4.1.2 Effect of Light and Photoperiod**

##### **4.1.2.1 Standard Photoperiod**

In the binary-choice experiments presenting adult vine weevils with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer arm with yew or weevil odours) under a 'Night' condition (Fig. 3, Experiment 1), the number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 6, no. trials = 13,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 10, no. trials = 17,  $p > 0.05$ ) (Fig. 3, Experiment 1). The number of weevils not responding to the choice between control and yew odour was 56.67 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 17 % of the introduced weevil population (Fig. 3, Experiment 1).

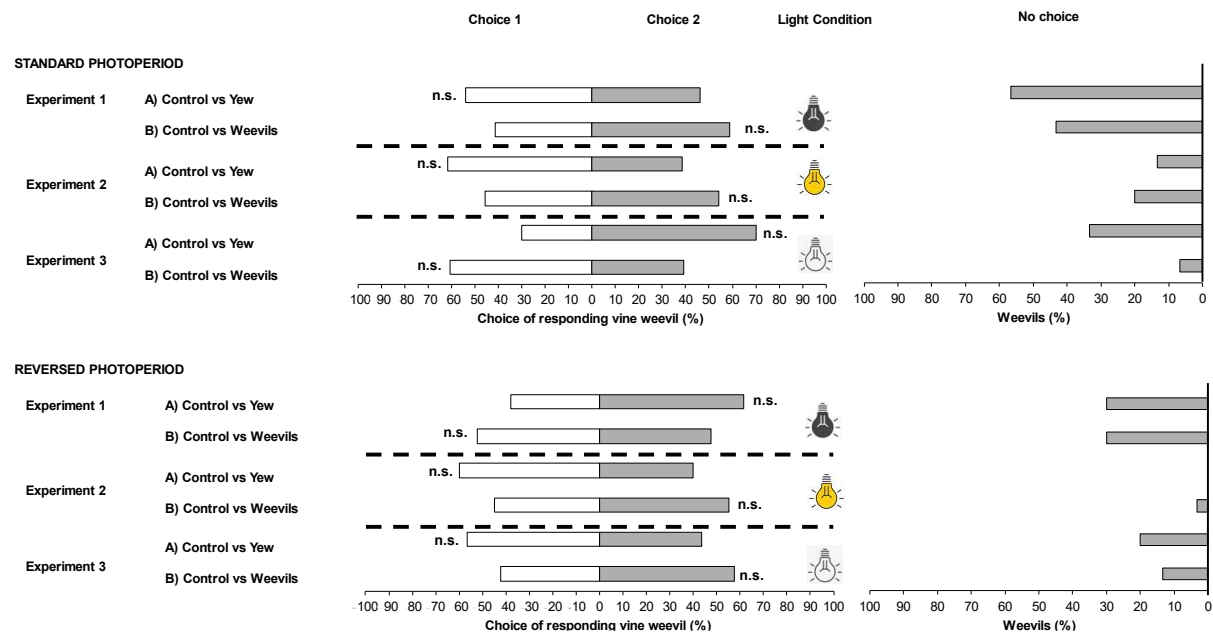
The number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 10, no. trials = 26,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 13, no. trials = 24,  $p > 0.05$ ) under a 'Day' condition (Fig. 3, Experiment 2). The number of weevils not responding to the choice between control and yew odour was 13.33 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 20 % of the introduced weevil population (Fig. 3, Experiment 2).

The number of weevils recorded in the treatments (yew or weevils) arm was not significantly different compared to that in the control arms (for treatment with yew: binomial

exact test: no. successes = 14, no. trials = 20,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 11, no. trials = 28,  $p > 0.05$ ) under a 'Dawn' condition (Fig. 3, Experiment 3). The number of weevils not responding to the choice between control and yew odour was 33.33 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 6.67 % of the introduced weevil population.

#### 4.1.2.2 Reversed Period

In the binary-choice experiments presenting adult vine weevils with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer arm with yew or weevil odours) under a 'Night' condition (Fig. 3, Experiment 1), the number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 13, no. trials = 21,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 10, no. trials = 21,  $p > 0.05$ ). The number of weevils not responding to the choice between control and yew odour was 30 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 30 % of the introduced weevil population (Fig. 3, Experiment 1).



**Figure 3:** Behavioural responses of vine weevil adults (%) toward two experimental treatments: (A) control and yew and (B) control and weevils. Each experimental treatment was tested under three different light conditions: Night (= 🌑 , Experiment 1), Day (= 🌞 , Experiment 2) and Dawn (= 🌅 , Experiment 3) and two photoperiods (Standard and Reverse). For each experimental treatment a single vine weevil adult (number of replicates = 30) was released in the release chamber of the Y- tube olfactometer. n.s., not significant.

The number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 12, no. trials = 30,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 16, no. trials = 29,  $p > 0.05$ ) under a 'Day' condition (Fig. 3, Experiment 2). The number of weevils not responding to the choice between control and yew odour was 3.33 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil odour was 0 % of the introduced weevil population (Fig. 3, Experiment 2).

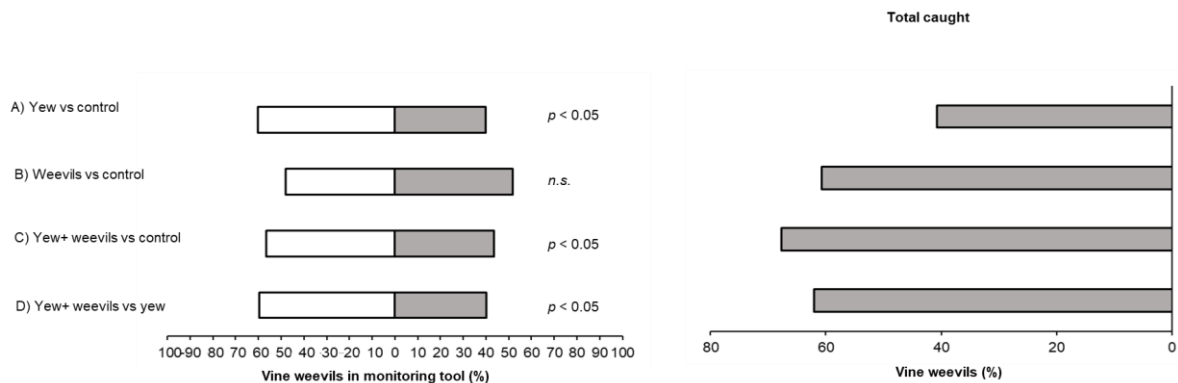
The number of weevils recorded in the treatment (yew or weevils) arms was not significantly different compared to that in the control arms (for treatment with yew: binomial exact test: no. successes = 10, no. trials = 23,  $p > 0.05$ ) (for treatment with weevils: binomial exact test: no. successes = 15, no. trials = 26,  $p > 0.05$ ) under a 'Dawn' condition (Fig. 3, Experiment 3). The number of weevils not responding to the choice between control and yew odour was 20 % of the introduced weevil population. The number of weevils not responding to the choice between control and weevil was 13.33 % of the introduced weevil population (Fig. 3, Experiment 3).

## **4.2 Glasshouse Experiments**

### **4.2.1 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance**

In a series of four binary-choice experiments adult vine weevils were presented with monitoring tools baited with combinations of the following: yew, conspecifics, yew + conspecifics or left unbaited. In the first experiment, 60 % of the individuals were recorded in monitoring tools baited with yew compared to 40 % in the ones left unbaited (binomial exact test: no. of successes = 98, no. of trials = 163,  $p = 0.01$ ). Total catch in this experiment was 40.75 % of the introduced weevil population (Fig. 4 A). In the second experiment, there was no significant difference between the number of weevils recorded in monitoring tools with 48.1 % retained in those baited with 20 conspecifics compared to 51.9 % in those left unbaited (binomial exact test: no. of successes = 117, no. of trials = 43,  $p = 0.6$ ). Total catch in this experiment was 60.7 % of the introduced weevil population (Fig. 4 B). In the third experiment, 56.5 % of individuals were recorded in monitoring tools baited with yew plus 20 conspecifics compared to 43.5 % in those left unbaited (binomial exact test: no. of successes = 153, no. of trials = 271,  $p = 0.03$ ). Total catch rate in this experiment was 67.8 % of the introduced weevil population (Fig. 4 C). In the final experiment, 59.7 % of individuals were recorded in monitoring tools baited with yew plus conspecifics compared to 40.3 % of those baited with only yew

(binomial exact test: no. of successes = 148, no. of trials = 248,  $p = 0.008$ ). Total catch in this experiment was 62 % of the introduced weevil population (Fig. 4 D).



**Figure 4.** Percentage of vine weevil adults recorded in monitoring tools under four experimental treatments: (A) yew and control, (B) weevils and control, (C) yew plus weevils and control, and (D) yew plus weevils and yew. For each experimental treatment (carried out under glasshouse conditions) vine weevil adults were released as a group of 40 individuals (number of replicate days = 5) (Binomial exact test:  $p < 0.05$ ); n.s., not significant.

## 5. Discussion

While in Chapter 2 vine weevil adults have been shown to respond positively to visual stimuli such as colour, diameter, height, *etc.*, it is important to also consider how olfactory cues may enhance monitoring tool efficacy. Volatile organic compounds isolated from either host plants or conspecifics are often used (Cano-Ramírez *et al.*, 2012; El-Shafie and Faleiro, 2017; Abd El-Ghany, 2019; García-Díaz *et al.*, 2020; Bandeira *et al.*, 2021) to significantly improve their sensitivity by enabling detection of the pest species even at low densities (Mahroof and Phillips, 2008). The results from this study show that monitoring tools baited with yew were preferred by vine weevil adults to unbaited monitoring tools. The response of vine weevil to host plant volatiles has been the focus of several studies (van Tol and Visser, 2002; van Tol *et al.*, 2002; 2004; Roberts *et al.*, 2019). For example, Roberts *et al.* (2019a) showed that vine weevil adults preferentially used refuges baited with Fortune's spindle (*E. fortune*) or yew (*T. baccata*) foliage compared to unbaited refuges, but that this was influenced by previous feeding experience of the adults. Although this species has a wide host range, it discriminates between the odours of potential host plants (van Tol *et al.*, 2002; Roberts *et al.*, 2019a). Further studies are needed to understand which plant volatiles elicit vine weevil responses and the correct ratio (Webster *et al.*, 2010) when using blends of plant volatiles to develop an effective semiochemical lure for monitoring this pest.

Plant volatiles are characterised by different bioactive chemical compounds that can be used to target more than one pest species and may be more practical and economical for

both monitoring and mass trapping than those for single species. For example, Sweeney *et al.* (2006) reported that a monitoring tool baited with a blend of monoterpenes, isolated from spruce (*Picea* spp.; Pinales: Pinaceae) and ethanol increased the probability of detecting not only the brown spruce longhorn beetle (*Tetropium fuscum* Fabricius; Coleoptera: Cerambycidae) but also other longhorn beetles. Host plant volatiles impact insect pests in different ways (El-Ghany, 2019) and may also play a role in modifying the behaviour of weevils (Reddy and Guerrero, 2004) as attractants, oviposition stimulants or as potential synergists for an aggregation pheromone (Tilles *et al.*, 1986; Tinzaara *et al.*, 2007).

Pheromone-based pest management strategies have been used as a tool for monitoring and mass trapping insect pests, including weevils (Bandeira *et al.*, 2021). Sex and aggregation pheromones have been identified in several insect pest species (Bandeira *et al.*, 2021) and attraction to a pheromone-based lure is typically species-specific. While vine weevil adults exhibit a strong tendency to aggregate, the mechanism underlying this behaviour has not, to date, been identified. van Tol *et al.* (2004) and Nakamuta *et al.* (2005) reported positive behavioural responses to conspecifics, while Pickett *et al.* (1996) suggested that previously occupied refuges stimulate aggregation behaviour. In this study the presence of weevils in Y-tube olfactometer bioassays as well as in monitoring tools did not significantly affect the response of conspecifics under laboratory and glasshouse conditions, respectively.

Pope and Roberts (2022) suggested that aggregation and conspecific attraction in this species may be mediated by frass or other nonchemical cues rather than by pheromones. Indeed, here it is shown that higher numbers of vine weevil adults were recorded in monitoring tools with yew and conspecifics than in monitoring tools left unbaited or containing only yew. This suggests that vine weevil adults may use volatiles associated with frass or induced by conspecifics during feeding as olfactory cues. Similar results have been reported for other weevil species (Barnes and Capatos, 1989; Sun *et al.*, 2010; Magalhães *et al.*, 2012). For example, the tea weevil (*Myloccerinus aurolineatus* Voss; Coleoptera: Curculionidae) has been shown to respond positively to tea plants infested with conspecifics but not to undamaged tea plants (Sun *et al.*, 2010). Similarly, Adesso *et al.* (2011) showed that the pepper weevil (*Anthonomus eugenii* Cano; Coleoptera: Curculionidae) preferred damaged flowering and fruiting plants over undamaged plants. In addition, the odour from flowering and fruiting plants with actively feeding weevils was preferred to the odour from plants with older feeding damage. The results presented here combined with knowledge of the olfactory responses of other species of weevils highlight the importance of identifying and exploiting herbivore-induced host plant volatiles for monitoring purposes.

In the last decades, day/night cycles, light/dark conditions and light quality have all been reported to regulate many aspects of plant physiology, growth and development (Webb, 2003). For example, it has been documented that the emission of volatile organic compounds

from plants is largely altered by the night/day cycles and light conditions (Colquhoun *et al.*, 2013; Giacomuzzi *et al.*, 2017). Herbivorous insects may be able to perceive these changes and use them as cues to adapt their behaviour accordingly (Kegge *et al.*, 2013). Light, therefore, may directly affect the physiology of the host plant and in turn the behaviour of herbivorous insects.

Greene and Morrill (1970) reported photonegative and photopositive responses in the behavioural orientation of the larvae of the cabbage looper (*Trichoplusia ni*, Hübner; Lepidoptera: Noctuidae) and the fall armyworm (*Spodoptera frugiperda*, J. E. Smith; Lepidoptera: Noctuidae), respectively. It has also been shown that the nocturnal pine weevil (*Hylobius abietis* L.; Coleoptera: Curculionidae) caused significant damages to three pine species (e.g., *Pinus sylvestris*, *Pinus pinaster* and *Pinus radiata*) (Pinales: Pinaceae) under reduced light conditions than under natural sunlight (López-Goldar *et al.*, 2016). Similarly, behaviour and movement of the alfalfa weevil (*Hypera postica* Gyllenhal; Coleoptera: Curculionidae) adults appear to be sensitive to light (Poinar and Gyrisco, 1964) regulating the movements of this species during the day. In particular, it has been observed that adults remain in debris on the ground or on the underside of lower dried-up leaves of alfalfa (Fabales: Fabaceae) plants during daylight hours. After sunset, adults become active and move away from the base of the tree, crawling up on plants to feed and remaining there all night.

In this study, no significant evidence was found, under the laboratory conditions used, to indicate that photoperiod affects vine weevil adult behaviour and its responses to host plant volatiles (yew). It is believed that vine weevil adults, under field conditions, are more active and feed on their hosts at night or twilight hours (van Tol *et al.*, 2012). However, it remains unclear whether vine weevil response to plant volatiles is due to a direct effect of the light and/or photoperiod, or, instead, due to other factors (e.g., impaired inducibility of plant defences in absence of light). Generally, the behavioural response of insects to plant volatiles is influenced by numerous factors, including sex, development stage, physiological state, degree of ecological specialization and feeding experience (Anderson and Anton, 2014). Therefore, to develop an effective semiochemical-based vine weevil management strategy using host plant volatiles requires further work to address each of these factors.

Insect response to odours derived from host plant and conspecific may vary depending on physiological factors such as mating status, nutritional and feeding status as well as sexual maturation (Gadenne *et al.*, 2016; Tasnin *et al.*, 2020; Roberts *et al.*, 2023). The age of an individual is another factor that has been shown to strongly affect the odour response of an insect (Reyes *et al.*, 2017). In this study, old (approximately ten-month-old) vine weevil adults tested in Y-tube olfactometer bioassays resulted to be, overall, less responsive to the stimuli tested (yew and conspecifics odours) than has been shown in previous work (Roberts *et al.*, 2019a, b). The responses of weevils to the tested stimuli (yew and conspecific odours) were

inconsistent throughout the experiments (Figures 2 and 3). Notably, few individuals crossed the mid-line of the olfactometer arms, with many simply sitting in the mouth of the Y-tube for the 20 minutes of the observation period. It is hypothesised that, similar to other insects, vine weevil olfactory responses to olfactory stimuli might decline with increasing age. Effect of age on response to host plant volatiles has been reported also for other weevils (Curculionidae), including the Plum Curculio (*Conotrachelus nenuphar* Herbst) the pepper weevil (*Anthonomus eugenii* Cano) (Addesso and McAuslane, 2009) the maize weevil (*Sitophilus zeamais* Motschulsky) and the granary weevil (*S. granarius* L.) (Wakefield, 1998). In another study, Arnold *et al.* (2012) reported that the olfactory behaviour and orientation of the cowpea weevil (*Callosobruchus maculatus* F.; Coleoptera: Chrysomelidae) towards infested and uninfested cowpeas (*Vigna unguiculata* (L.) Walp.; Fabales: Fabaceae) were influenced by age. Similarly, orientation to odour sources by the Queensland fruit fly (*Bactrocera tryoni* Froggatt; Diptera: Tephritidae) has been shown to be lower at fifteen weeks compared to three weeks (Tasnin *et al.*, 2020). As olfactory response of an insect pest may vary with age, future research is needed to understand how age and other physiological factors can affect olfactory responses of vine weevil, which will allow to improve the use of olfactory stimuli for controlling and managing this pest.

To conclude, olfactory cues (e.g. yew, yew+ weevils) have been shown to improve vine weevil monitoring tool efficacy. Although efforts are being made to identify an effective semiochemical lure for vine weevil, further studies need to be completed to identify and include these olfactory cues in monitoring tools. For an effective monitoring system to be developed for vine weevil adults, knowledge of the factors that may influence as well as the mechanisms involved in the attraction of this insect to its host and conspecifics is crucial.

# Chapter 4: Effect of Apple Volatiles on Vine Weevil Adults Behaviour<sup>3</sup>

## Abstract

Volatile organic compounds (VOCs) derived from apple (*Malus* spp.; Rosales: Rosaceae) have been used as a lure to enhance monitoring tool efficacy for economically important insect pests (Bengtsson *et al.*, 2001; Coracini *et al.*, 2004). This study investigated the electrophysiological and behavioural responses of vine weevil adults (*Otiorhynchus sulcatus* F.; Coleoptera: Curculionidae) to volatiles released from apple sauce using chemical analysis by gas chromatographic coupled electroantennographic analysis as well as Y-tube olfactometer bioassays. The behavioural responses of adult vine weevils to olfactory cues derived from three apple products (sauce, vinegar and chips) were also tested under glasshouse conditions. Volatiles released from apple sauce affected the behaviour of vine weevil adults with more adults selecting the Y-tube olfactometer arm containing apple volatiles (0.1 g, 1 g and 10 g) compared to the control arm. However, only weak electroantennographic responses were observed to a range of VOCs released from apple sauce. Under glasshouse conditions, a greater number of vine weevils were recorded in monitoring tools baited with apple vinegar or sauce compared to unbaited monitoring tools. Baiting monitoring tools with apple chips did not increase the number of vine weevils recorded in monitoring tools. In addition, monitoring tools baited with apple sauce and spindle (*Euonymus fortunei* (Turcz.) Hand.-Maz.; Celastrales Celastraceae) had higher numbers of vine weevils than monitoring tools left unbaited or those baited with only spindle or apple.

This study suggests that apple sauce influences vine weevil behaviour and could serve as a basis for developing a new lure to enhance vine weevil monitoring tool efficacy.

## 1. Introduction

Fruit-based volatiles have been widely reported to enhance monitoring tool efficacy as well as to offer an effective and environmentally benign approaches for monitoring and controlling insect pest populations (Bolton *et al.*, 2019; Tasnin *et al.*, 2020). Blueberries (*Vaccinium corymbosum* L.; Ericales: Ericaceae) and tart cherries (*Prunus cerasus* L.;

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<sup>3</sup> Part of the data presented in this Chapter (GC-MS and GC-EAD) was obtained in an experiment carried out by Dr Steven Harte, Prof David Hall and Dr Daniel Bray from the University of Greenwich (UK).

Rosales: Rosaceae) significantly increased capture rates for the Blueberry maggot flies (*Rhagoletis mendax* Curran; Diptera: Tephritidae) and the Eastern cherry fruit fly (*R. cingulata* Loew) on adjacent sticky traps compared to traps not baited with fruits (Pelz-Stelinski *et al.*, 2005). Similarly, the use of traps baited with orange juice caught higher numbers of Mediterranean fruit fly (*Ceratitis capitata*, Wiedemann; Diptera: Tephritidae) compared to traps baited with other natural fruit juice-based lures (Suárez *et al.*, 2023). Therefore, these bioactive natural compounds derived from fruits could play an important role in developing effective monitoring tools to enhance integrated pest management (IPM) programmes (Natale *et al.*, 2004; Stuhl, 2021).

Apple (*Malus domestica* Borkh; Rosales: Rosaceae) is one of the most widely cultivated fruit crops in temperate regions (Brown, 2012). Apples originated from a wild apple species (*Malus sieversii* Lebed.), which is native to the mountainous regions of Central Asia, in what is today known as Kazakhstan (Musacchi and Serra, 2018). Volatile organic compounds (VOCs) derived from apple (*Malus* spp.; Rosales: Rosaceae), especially those emitted by flowers or ripe fruit, have been widely used as lures (Bengtsson *et al.*, 2001; Coracini *et al.*, 2004) to enhance monitoring tool efficacy. It has been shown that the Australian sap beetle (*Carpophilus davidsoni* Dobson; Coleoptera: Nitidulidae) responds to odours from ripening fruit or fermenting foods (e.g., fermenting apple juice) and these volatiles have been successfully used for population monitoring (Bartelt and Hossain, 2006). In addition, several studies have reported the use of apple-baited monitoring tools to monitor spotted wing drosophila (*Drosophila suzukii* Matsumura; Diptera: Drosophilidae) (Feng *et al.*, 2018; Cai *et al.*, 2019).

Apple-derived volatiles have been reported to be attractive also to weevil (Curculionidae) pests (Smith, 1932). For example, Leskey and Prokopy (2000) reported positive behavioural responses in the plum curculio (*Conotrachelus nenuphar* Herbst; Coleoptera: Curculionidae) to volatiles derived from the McIntosh red apple variety (*Malus domestica* McIntosh; Rosales: Rosaceae). Melander and Spuler (1926) used apple pomace as bait to coat inorganic insecticides (e.g., zinc arsenite, magnesium arsenate or calcium arsenate) to control the strawberry root weevil (*Otiorhynchus ovatus* L.; Coleoptera: Curculionidae) adults. Similarly, Mote and Wilcox (1927) reported the use of an apple-based bait to increase the efficacy of a killing agent used against the strawberry root weevil and the rough strawberry weevil (*O. rugosostriatus* Goeze; Coleoptera: Curculionidae). Li *et al.* (1995) tested the use of apple pomace to increase the efficacy of grooved board trap on capturing vine weevil adults in raspberries (*Rubus idaeus* L; Rosales: Rosaceae).

Vine weevil, similar to other insect pests, uses visual cues for orientation (Chapter 2) but most likely also relies on olfactory cues to discriminate between host and non-host plants (Bjorklund *et al.*, 2005; Adesso and McAuslane, 2009). Although the use of apple as lure to

attract weevils has been explored for some species (Melander and Spuler, 1926; More and Wilcox, 1927; Smith, 1932; Lie *et al.*, 1995), direct evidence for the attraction of vine weevils to fruit-odour volatiles, such as apple volatiles, remains limited. However, apple chips are used in ChemTica traps (ChemTica's personal communication) for attracting vine weevils, suggesting that apple-derived volatiles may play a role in their olfactory preferences. Therefore, as part of a programme to develop an effective vine weevil monitoring tool, this study reports the results of laboratory and glasshouse experiments designed to: (i) investigate the behavioural responses of vine weevil adults to volatiles originating from apple sauce under laboratory condition; (ii) evaluate the efficacy of a model monitoring tool baited with apple-based volatiles under glasshouse condition. In addition, gas chromatography coupled mass spectrometry (GC-MS) and gas chromatography coupled electroantennographic detection (GC-EAD) were used as a first step toward the identification of the behaviourally active compounds originating from apple sauce as well as to investigate vine weevil responses to these fruit-based volatiles. It is hypothesised that volatiles emitted by apple-based products significantly affect the behavioural responses of adult vine weevils relative to the source of this stimulus.

## **2. Materials and Methods**

### **2.1 Insects**

Adult vine weevils were collected from commercial strawberry (*Fragaria x ananassa* cv. Duchesne) crops in Norfolk (UK) during summer 2023. These individuals were maintained in plastic terraria (30 × 19.3 × 20.6 cm; Exo Terra, Castleford, UK) containing spindle (*Euonymus fortunei* (Turcz.) Hand.-Maz.; Celastrales: Celastraceae) foliage with moist paper towels as a food and water source with cotton wool balls to act as an oviposition site. Spindle foliage, paper towels and cotton wool balls were replaced weekly. These terraria were housed in a controlled environment room maintained at 20 °C and 60 % relative humidity with a 16:8 light:dark photoperiod (Fitotron, Weiss Technik, Ebbw Vale, Wales, UK). Eggs were collected every three days from the cotton wool balls in each terrarium using a fine 000 paintbrush. Collected eggs were maintained in plastic containers (17 x 10.2 x 3 cm, GP Globe Packaging, UK) filled with compost (SylvaGrow® John Innes Number 2, Tetbury, UK) within a controlled environment room (20 °C, 60% relative humidity; Fitotron) under complete darkness to produce larvae. Carrot slices were placed on the surface of the growing media each week as a food source for emerging larvae. Larvae that completed their development were maintained as adults under the previously stated environmental conditions using a standard method of

placing the weevils in plastic terraria containing spindle foliage and moist paper towels that were replaced weekly. All adult weevils that were used in the following experiments were at least three months old and reproductively active.

## **2.2 Laboratory Experiments**

### **2.2.1 Collection of Apple Sauce Volatiles**

Apple volatiles were collected from 30 g of apple sauce (Bramley apple sauce; Tesco Stores Ltd., UK). These were placed in a 5-liter round-bottom bolt-head flask, and air was drawn into the flask at a rate of 2 l/min through a filter containing activated charcoal (20 cm x 1.5 cm; 6-10 mesh) and out through a filter made from a Pasteur pipette (4 mm internal diameter (i.d.)), which contained 200 mg of Porapak Q (50-80 mesh; Supelco, Gillingham, Dorset, UK) packed between two silanized glass wool plugs. Volatile compounds were collected over 3 hours. Trapped volatiles were eluted using 1 ml of dichloromethane (Pesticide Residue Grade) and stored at 4 °C until analysis.

### **2.2.2 Analysis by GC-MS**

Analyses were carried out using a 6890N gas chromatograph (GC) coupled to a 5973 Ion Trap Detector (Agilent Technologies, Cheadle, UK). The GC was fitted with a fused silica capillary column (30 mm × 0.25 mm × 0.25 µm film thickness) coated with DBWax (Supelco, Bellefonte, PA). Manual injections of 1 µl were made in splitless mode at 240 °C. The column oven was programmed to increase from 40 °C (2 min delay) to 250 °C at a rate of 10 °C min<sup>-1</sup>. Compounds were identified based on their mass spectrum, retention indices compared to n-alkanes and co-chromatography with authentic compounds.

### **2.2.3 Analysis by GC-EAD**

Analyses were carried out using a HP6890 gas chromatograph (GC) (Agilent Technologies) fitted with a flame ionization detector (FID) and fused silica capillary columns (30 m × 0.25 mm × 0.32 µm film thickness) coated with DBWax and DB1 (Supelco). Injections onto the DBWax column were made in splitless mode at 220 °C, with the column oven temperature programmed from 50 °C (2 min delay) to 250 °C at a rate of 20 °C min<sup>-1</sup> for analyses. The effluents from both columns were combined using a glass push-fit Y-tube

connector (Agilent Technologies), linked to another Y-tube connector via deactivated fused silica tubing (10 cm × 0.32 mm i.d.). One arm of this connector was connected to the FID (at 250 °C) using 50 cm of fused silica tubing (0.32 mm i.d.), while the other arm led through a heated transfer line (250 °C; Syntech, Hilversum, now Kirchzarten, Germany) into a glass tube (4 mm i.d.), where air (500 ml/min) passed over the EAG preparation.

Electroantennogram (EAG) recordings were performed using a portable INR-02 device (Syntech) connected as a second detector of the GC for analog-to-digital (A/D) conversion. Glass electrodes containing an electrolyte solution (0.1 M potassium chloride with 1% polyvinylpyrrolidone) were mounted onto silver wires held in micromanipulators. Vine weevils were anesthetized using carbon dioxide before excising their antenna using a scalpel. The reference electrode was inserted into the base of the antenna, while the circuit was completed by placing the recording electrode in contact with the tip of one antenna. Both the flame ionization detector (FID) and EAG signals were recorded and analysed using EZChrom software (Elite v3.0; Agilent Technologies).

#### **2.2.4 Y-tube Olfactometer Bioassay**

The behavioural responses of adult vine weevils to olfactory stimuli were tested using the same glass Y-tube olfactometer (Sci-Glass Consultancy, Bere Alston, UK) described in Chapter 3 and based upon the design previously employed by Roberts *et al.* (2019b; 2023). The airflow was purified by passing it through a charcoal filter, an air humidifier (tap water) and then passed through two serially connected Drechsel bottles (500 ml) and connecting arms before entering the Y-tube. The airflow was set to 1200 ml.min<sup>-1</sup> with odour sources held in the first set of Drechsel bottles for at least one hour before an experiment began. An empty Drechsel bottle was used for the control. All bioassays were undertaken in a controlled environment room (20 °C, 60% relative humidity; Fitotron) between 07:00 and 11:00 in the morning. Within this controlled environment, light emitted by fluorescent tube (Philips Master TL-D 70W/840, UK) was set up to simulate light levels at dawn (138 Lux).

The olfactory stimuli used in the Y-tube bioassays comprise apple sauce (Bramley apple sauce; Tesco Stores Ltd., UK) at different doses (0.1 g, 1 g, 10 g, 20 g) or small branches of spindle (10 or 20 g, Ø ~ 1 cm and length ~ 10 cm) previously identified as eliciting a response to vine weevil adults (Roberts *et al.*, 2019). Freshly cut branches of spindle were collected from the Harper Adams University grounds and used in the experiments within four hours of collecting.

Prior to use in a bioassay, vine weevils were starved for forty-eight hours. A single vine weevil (approximately three-month-old) was introduced into the olfactometer via a release

chamber using a fine brush. A choice was recorded when the weevil reached the collecting point at the end of the branched arms. Non-responding adults were recorded as such. Each pair of odour sources (e.g., apple sauce or spindle vs. control or spindle) was tested twenty times using fresh individuals and the number of weevils that reached the end of each arm within the 20-minute testing period was recorded. To minimize any potential directional bias, the position of the odour sources was alternated between replicates. After each test, all glassware was thoroughly cleaned by rinsing with warm water followed by HPLC-grade acetone (Sigma Aldrich) and then baked in a glassware oven at 120 °C for 15 min (Roberts *et al.*, 2019b).

## **2.3 Glasshouse Experiments**

### **2.3.1 Experimental Set-up**

Experiments testing vine weevil behaviour towards a monitoring tool with or without olfactory cues were carried out in a glasshouse representing a semi-field environment. This semi-field environment was created using potted ( $\varnothing = 13$  cm; Teku VCH13, Pöppelmann, Lohne, Germany) strawberry plants (*cv. Elsanta*; RW Walpole, King's Lynn, UK) placed in a fine mesh tent cage (145 × 145 × 152 cm) (Insectopia, Austrey, UK) situated within a glasshouse (mean temperature  $19.2 \pm 0.8$  °C, mean humidity  $52.2 \pm 1.6\%$ ). Four potted strawberry plants were positioned equidistant from one another along the perimeter of a 110 cm<sup>2</sup> square centrally positioned within the tent cage as described in Chapter 3, providing both a food source and alternative refuges. Monitoring tools were created from paper cups (height = 11.3 cm,  $\varnothing$  = base 5.8 cm,  $\varnothing$  rim = 8.9 cm) (Comfy Package, New York, US), externally and internally painted black using poster paint (Galeria Acrylic, Windsor & Newton, London, UK). Paper cups were inverted so that the rim became the monitoring tool base and four equally distanced entrances were made in the monitoring tool by cutting 1 cm<sup>2</sup> openings around the cup rim. A roll of corrugated card (length 30 cm, width 3 cm) was inserted into each refuge to provide shelter by exploiting the thigmotactic behaviour exhibited by this species.

### **2.3.2 Effect of Olfactory Cues on Vine Weevil Monitoring Tool Performance**

The behavioural response of adult vine weevils to olfactory cues was tested in seven binary-choice experiments under the glasshouse conditions as previously described in Section 2.3.1. Small branches of spindle (*E. fortunei*) ( $\varnothing \sim 1$  cm and length  $\sim 4$  cm) or apple chips (Tesco Stores Ltd., UK) were placed in white organza bags (7 × 9 cm; OWill, UK), whereas

apple sauce or apple vinegar (Aspall Cyder House, UK) were contained in Petri dishes (35 x 10 mm; Sarstedt AG & Co. KG, Germany). A drill (Dremel 3000, Eternal Tools, UK) was used to make a 1 cm hole in the lid of these Petri dishes and a fine mesh was glued over this hole to prevent direct contact between the adults and the treatments. Olfactory stimuli were then placed inside a monitoring tool. For Experiment 1 (Table 1) two monitoring tools were baited with 5 g of apple vinegar and two contained an empty Petri dish (35 x 10 mm). For Experiment 2 (Table 1) two monitoring tools were baited with 5 g of apple chips and two contained an empty organza bag. For Experiment 3 (Table 1) two monitoring tools were baited with 5 g of apple sauce and two contained an empty Petri dish. In Experiment 4 (Table 1) two monitoring tools were baited with 5 g of apple sauce and two monitoring tools were baited with 5 g of spindle. For Experiment 5 (Table 1) two monitoring tools were baited with 5 g of apple sauce plus 5 g of spindle and two contained an empty Petri dish plus empty organza bag. In Experiment 6 (Table 1) two monitoring tools were baited with 5 g of apple sauce plus 5 g of spindle and two monitoring tools were baited with 5 g of spindle. In Experiment 7 (Table 1) two monitoring tools were baited with 5 g of apple sauce plus 5 g of spindle and two monitoring tools were baited with 5 g of apple sauce.

Monitoring tools and the tent cage (n = 2) to which monitoring tools were allocated were re-randomised each day to exclude the effect of position. Vine weevil populations, along with the monitoring tools and odour sources, were replaced between each replicate. Forty adult vine weevils were collected from the laboratory culture, placed in a plastic box ( $\varnothing = 12$  cm) and then released into the centre of the experimental arena between 17:00 and 20:00. The location of each vine weevil was recorded between 08:00 and 09:00 the following day. For each binary choice experiment (Table 1), a total of 400 individuals were tested (two replicates per day for 5 days).

**Table 1.** Experiments testing the effect of olfactory cues on vine weevil monitoring tool performance.

Experiment	Monitoring tool 1	Monitoring tool 2	No. of replicates
1	Unbaited	Apple vinegar <sup>a</sup>	5
2	Unbaited	Apple chips <sup>b</sup>	5
3	Unbaited	Apple sauce <sup>c</sup>	5
4	Apple sauce	Spindle <sup>d</sup>	5
5	Unbaited	Apple sauce + Spindle	5
6	Apple sauce + Spindle	Spindle	5
7	Apple sauce + Spindle	Apple sauce	5

<sup>a</sup> Apple vinegar (5 g) was used for this experiment.

<sup>b</sup> Apple chips (5 g) was used for this experiment.

<sup>c</sup> Apple sauce (5 g) was used for this experiment.

<sup>d</sup> Small branches of spindle (*Euonymus fortunei*) (5 g,  $\varnothing \approx 1$  cm and length  $\approx 4$  cm) were used for the experiments with spindle.

### 3. Statistical Analyses

All statistical analyses were carried using R (Version 4.2.2) (R Core Team, 2022). Y-tube olfactometer bioassays data were analysed using an exact binomial test against the null hypothesis that the number of vine weevils reaching the end of olfactometer arm had a 50:50 distribution (Roberts *et al.*, 2019; 2023). For binary-choice experiments carried out under glasshouse conditions the number of individuals within a monitoring tool (i.e., monitoring tool performance) was analysed also using an exact binomial test against the null hypothesis that the number of vine weevils seeking refuge had a 50:50 distribution (Roberts *et al.*, 2019a, b; 2023). Replicates from each experiment were pooled prior to analysis and non-responding individuals excluded.

## 4. Results

### 4.1 Laboratory Experiments

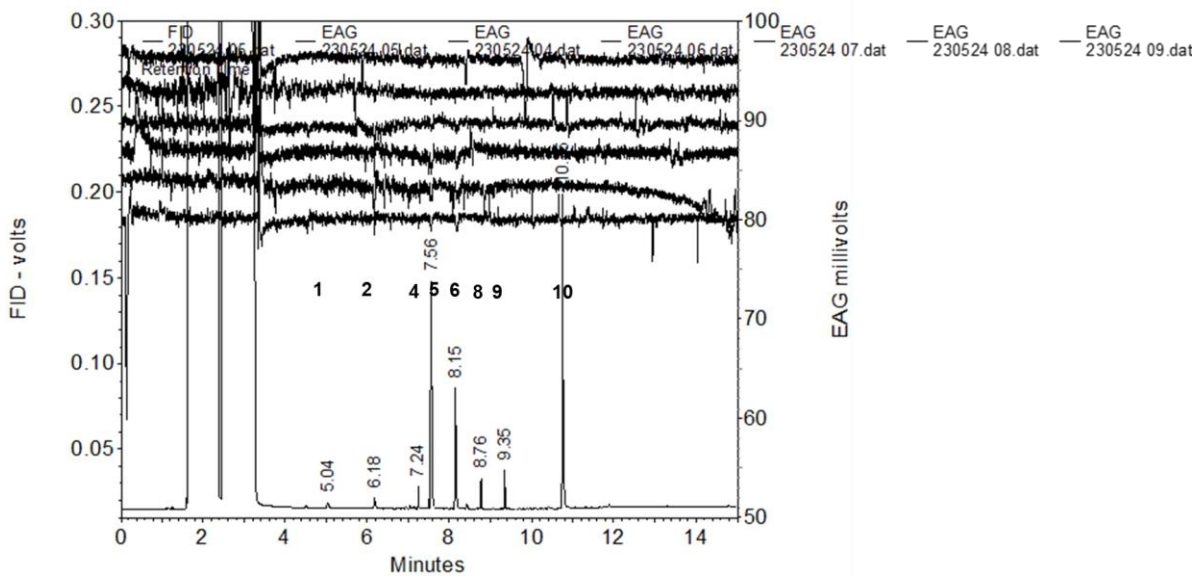
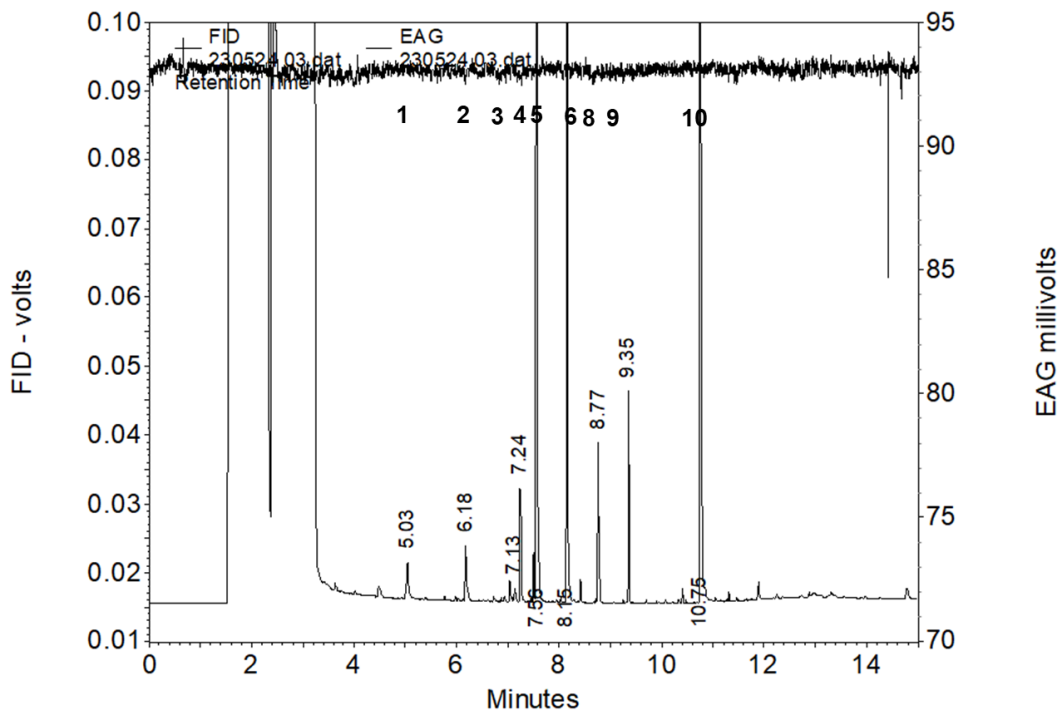
#### 4.1.1 GC/EAG and GC/MS Analyses of Apple Sauce Volatiles

From the analysis of volatiles emitted by apple sauce using GC-EAG and a vine weevil antenna, ten reproducible responses to volatiles from apple sauce were observed (Fig. 1; Table 2). The compounds' identities are presented in Table 2 along with their relative proportions in the samples used for GC-EAG analyses. There were weak EAG responses (e.g., EAG deflection in response to a particular compound is smaller compared to those exhibited by other compounds; Dr Daniel Bray personal communication) to (*S*)-2-methyl-1-butanol, hexanol, ethyl hexanol, decanal, octanol, (*E*)-2-decenal, salicylaldehyde, decanol, (*E*)-2-dodecenal and scorbic acid (Fig. 1).

**Table 2.** Compounds identified in gas chromatography coupled mass spectrometry (GC-MS) analyses of volatiles from Apple sauce volatile and relative amounts (EAG responses numbered as in Fig. 1).

EAG Response	RT (min)	KI	Peak Area (mAu*min)	Compound
1	5.03	1104	13313	( <i>S</i> )-2-methyl-1-butanol
2	6.18	1179	16574	Hexanol
3	7.13	1297	5419	Ethyl hexanol
4	7.24	1314	22530	Decanal
5	7.56	1363	33244	Octanol
6	8.15	1508	129025	( <i>E</i> )-2-decenal
7	8.42	1591	6217	Salicylaldehyde
8	8.76	1724	35240	Decanol
9	9.35	1876	36050	( <i>E</i> )-2-dodecenal
10	10.75	2157	415467	Scorbic acid

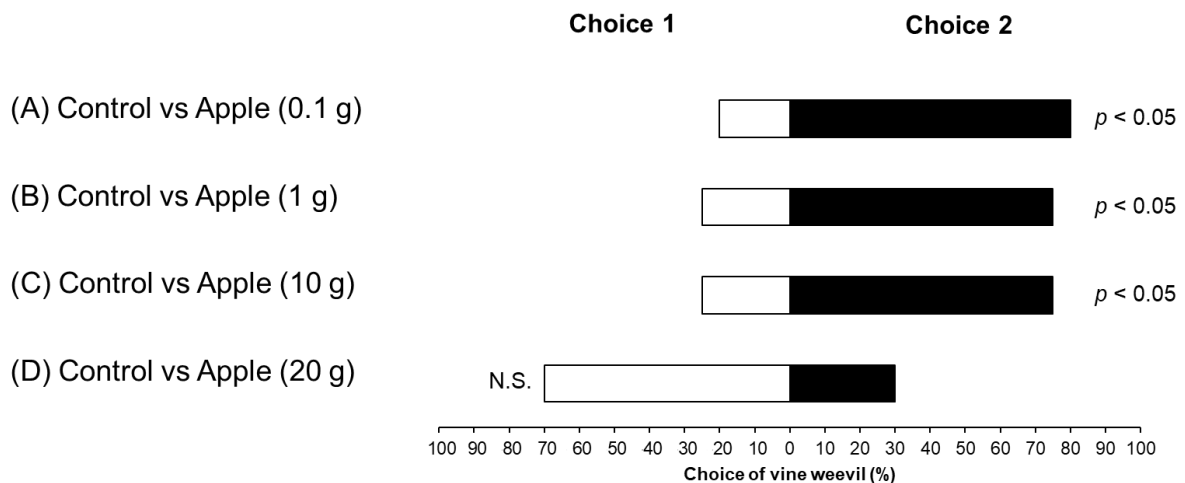
**RT:** Retention time; **KI:** Kovats retention indices relative to the retention times of n-alkanes on polar GC column. **mAU\*min:** Gas abundance (mAU) versus Retention time (minutes).



**Figure 1.** Gas chromatography/ electroantennogram (GC/EAG) analysis of volatiles from Apple sauce on polar GC column (responses numbered as in Table 2) (Source: Dr Steven Harte, Prof David Hall and Dr Daniel Bray from the University of Greenwich, UK).

#### 4.1.2 Vine Weevil Dose-Responses to Apple Sauce

In a series of binary-choice experiments vine weevil adults were presented with a choice between control and apple sauce at different doses (0.1 g, 1 g, 10 g and 20 g). In the first experiment, vine weevils exhibited a preference for the Y-tube olfactometer arm containing apple sauce at dose of 0.1 with 80 % of responding individuals choosing this arm over the clean-air control arm (binomial exact test: no. of successes = 16, no. of trials = 20,  $p < 0.05$ ) (Fig. 2A). In the second experiment, vine weevils exhibited a preference for the Y-tube olfactometer arm containing apple sauce at dose of 1 g with 75 % of responding individuals choosing this arm over the clean-air control arm (binomial exact test: no. of successes = 15, no. of trials = 20,  $p < 0.05$ ) (Fig. 2B). In the third experiment, vine weevils exhibited a preference for the Y-tube olfactometer arm containing apple sauce at dose of 10 g with 75 % of responding individuals choosing this arm over the clean-air control arm (binomial exact test: no. of successes = 15, no. of trials = 20,  $p < 0.05$ ) (Fig. 2C). In the fourth experiment, there was no preference between the Y-tube clean-air control arm with 70 % of responding individuals choosing this arm over the olfactometer arm containing apple sauce at dose of 20 g (binomial exact test: no. of successes = 14, no. of trials = 20,  $p = 0.12$ ) (Fig 2D).

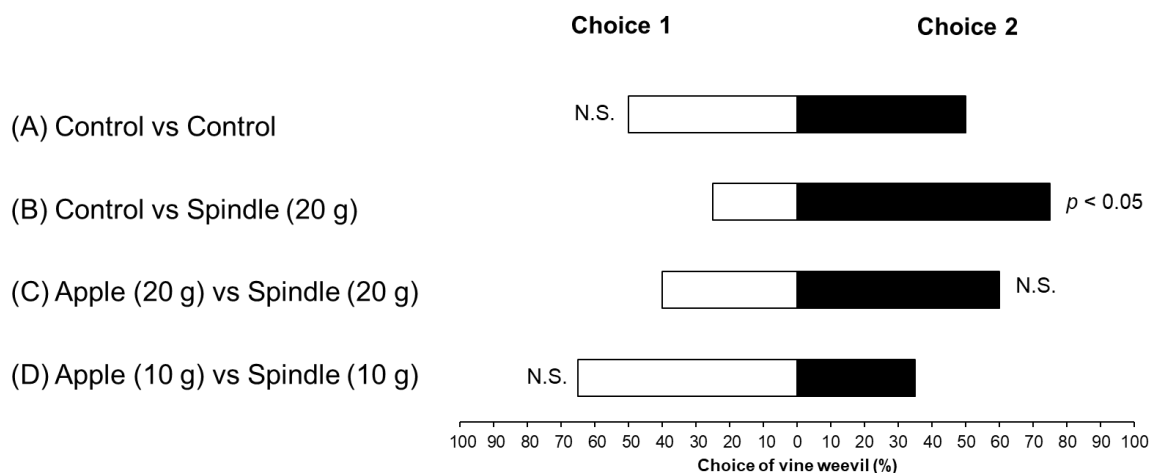


**Figure 2.** Behavioural responses of vine weevil adults towards apple sauce at different concentrations 0.1 g (A), 1 g (B), 10 g (C) and 20 g (D) when presented with control in a Y- tube olfactometer. For each experimental treatment 20 vine weevil adults were tested (binomial exact test:  $p < 0.05$ ); N.S. = not significant.

#### 4.1.3 Vine Weevil Response to Olfactory Cues

In a series of binary-choice experiments vine weevil adults were presented with a choice between control (empty Y-tube olfactometer arm) and treatments (Y-tube olfactometer

arm with spindle or apple sauce odours). In the first experiment, there was no preference between the two Y-tube olfactometer clean-air control arms (binomial exact test: no. of successes = 10, no. of trials = 20,  $p > 0.05$ ) (Fig. 3A). In the second experiment, vine weevils exhibited a preference for the Y-tube olfactometer arm containing spindle (*E. fortunei*) at dose of 20 g with 75 % of responding individuals choosing this arm over the clean-air control arm (binomial exact test: no. of successes = 15, no. of trials = 20,  $p = 0.04$ ) (Fig. 3B). In the third experiment, there was no preference between the Y-tube olfactometer arm containing spindle at dose of 20 g with 60 % of responding individuals choosing this arm over the arm containing apple sauce at dose of 20 g (binomial exact test: no. of successes = 12, no. of trials = 20,  $p = 0.5$ ) (Fig. 3C). In the fourth experiment, there was no preference between the Y-tube olfactometer arm containing apple sauce at dose of 10 with 65 % of responding individuals choosing this arm over the arm containing spindle at dose of 10 g (binomial exact test: no. of successes = 13, no. of trials = 20,  $p = 0.26$ ) (Fig. 3D).

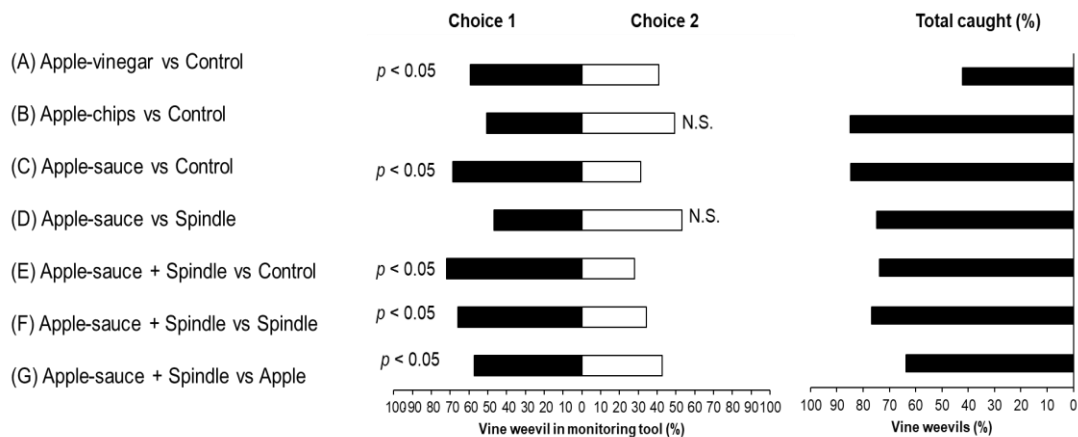


**Figure 3.** Behavioural responses of vine weevil adults in a Y-tube olfactometer under four experimental treatments: (A) control and control, (B) control and spindle (20 g) (*E. fortunei*), (C) apple sauce (20 g) and spindle (20 g) and (D) apple sauce (10 g) and spindle (10 g) (*E. fortunei*). For each experimental treatment 20 vine weevil adults were tested (binomial exact test:  $p < 0.05$ ); N.S. = not significant.

## 4.2 Glasshouse Experiments

In a series of binary-choice experiments adult vine weevils were presented with monitoring tools baited with combinations of the following: apple vinegar, apple chips, apple sauce, apple sauce + spindle or left unbaited. In Experiment 1, 59.17 % of individuals were recorded in monitoring tools baited with apple vinegar compared to 40.83 % in those left unbaited (binomial exact test: no. of successes = 100, no. of trials = 169,  $p = 0.02$ ) (Fig. 4A).

In Experiment 2, there was no significant difference between the number of weevils recorded in monitoring tools with 50.57 % retained in those baited with apple chips compared to 49.41 % in those left unbaited (binomial exact test: no. of successes = 172, no. of trials = 340,  $p = 0.87$ ) (Fig. 4B). In Experiment 3, 68.73 % of individuals were recorded in monitoring tools baited with apple sauce compared to 31.27 % in those left unbaited (binomial exact test: no. of successes = 233, no. of trials = 339,  $p < 0.05$ ) (Fig. 4C). In Experiment 4, there was no significant difference between the number of weevils recorded in monitoring tools with 46.82 % retained in those baited with apple sauce compared to 53.17 % in those baited with spindle (binomial exact test: no. of successes = 140, no. of trials = 299,  $p = 0.29$ ) (Fig. 4D). In Experiment 5, 71.86 % of individuals were recorded in monitoring tools baited with apple sauce plus spindle compared to 28.14 % in those left unbaited (binomial exact test: no. of successes = 212, no. of trials = 295,  $p < 0.05$ ) (Fig. 4E).



**Figure 4.** Percentage of vine weevil adults recorded in monitoring tools under seven experimental treatments: (A) apple-vinegar and control, (B) apple-chips and control, (C) apple sauce and control, (D) apple sauce and spindle (*E. fortunei*), (E) apple sauce plus spindle and control, (F) apple sauce plus spindle and spindle and (G) apple sauce plus spindle and apple. For each experimental treatment vine weevil adults were released as a group of 40 individuals (number of replicate days = 5) (binomial exact test:  $p < 0.05$ ); N.S. = not significant.

In Experiment 6, 65.79 % of individuals were recorded in monitoring tools baited with apple sauce plus spindle compared to 34.20 % in those with only spindle (binomial exact test: no. of successes = 202, no. of trials = 307,  $p < 0.05$ ) (Fig. 4F). In Experiment 7, 57.25 % of individuals were recorded in monitoring tools baited with apple sauce plus spindle compared to 42.74 % of those baited with only apple sauce (binomial exact test: no. of successes = 146, no. of trials = 255,  $p < 0.05$ ) (Fig. 4G).

## 5. Discussion

Phytophagous insects rely on olfaction as the principal sensory modality for sensing their external environment and mediating changes in their behaviour (e.g., mate selection, food choice, *etc.*) (Hansson, 1999). Attraction of insects to fruit-derived volatiles, which involves detection of blends or single compounds, has been exploited for both monitoring and direct control of insect pest populations (El-Ghany, 2019). In this study, the behavioural responses of vine weevil adults to apple-derived volatiles were investigated to determine their potential use as lure to improve monitoring of this species. Overall, the results showed that, volatiles derived from apple sauce elicit positive behavioural responses in vine weevil adults under both laboratory and glasshouse conditions. Vine weevil adults exhibited a preference for the Y-tube olfactometer arm containing apple sauce at dose of 0.1 g, 1g and 10 g over the clean-air control arms. Similarly, monitoring tools baited with apple sauce recorded 68.73 % of individuals compared to 31.27 % in those left unbaited. These results are in line with previous studies, where insect species have shown positive behavioural responses to apple volatiles. For example, the heaven root weevil (*Eucryptorrhynchus scrobiculatus* Motschulsky; Coleoptera: Curculionidae) and the apple maggot (*Rhagoletis pomonella* Walsh; Diptera: Tephritidae) have been reported to respond positively to apple odours (Zhang *et al.*, 1999; Yang *et al.*, 2019). Similarly, apple volatiles have shown to attract the oriental fruit moth (*Cydia molesta* Busck; Lepidoptera: Tortricidae) females (Natale *et al.*, 2004).

Although volatiles derived from apple sauce elicited positive behavioural responses in vine weevil adults under both laboratory and glasshouse conditions, the EAG response results indicated only weak responses to ten compounds identified from apple sauces: (*S*)-2-methyl-1-butanol, hexanol, ethyl hexanol, decanal, octanol, (*E*)-2-decenal, salicylaldehyde, decanol, (*E*)-2-dodecanal and scorbic acid. It is suggested that although some compounds might elicit weak antennal responses, they might still play a role in behavioural attraction or deterrence (Bruce *et al.*, 2005; Webster *et al.*, 2010). Furthermore, it is believed that there might be a potential involvement of other sensory modalities (e.g., gustatory or visual cues) or an interaction between volatiles in a natural context (Riffell *et al.*, 2008). Therefore, further investigations are needed to provide insight into vine weevil olfactory system's sensitivity to apple sauce compounds.

Insect behavioural responses to olfactory cues are affected by biotic and abiotic environmental factors, chemical and physical conditions of the host plant (Binyameen *et al.*, 2021) as well as physiological and motivational state of the insect (Gadenne *et al.*, 2016). The specific ratios and the combination of compounds in plant volatile blends are considered also to influence the interactions between plant hosts and phytophagous insects (Najar-Rodriguez *et al.*, 2010). Several dose-response studies have reported the importance of odour stimulus

concentration in insect responsiveness. For example, Webster *et al.* (2010), investigating the behavioural responses of the black bean aphid (*Aphis fabae* Scopoli, Hemiptera: Aphididae) to different doses of volatile compounds emitted by its host, the broad bean (*Vicia faba* L.; Fabaceae: Fabales), showed that 1-hexanol and (*Z*)-3-hexen-1-ol elicited a positive behavioural response only at the highest dose tested (100 ng). In another study, terpenes at lower doses, elicited positive behavioural responses in the African malaria mosquito (*Anopheles gambiae* Giles; Diptera: Culicidae) females, while at higher doses, avoidance behaviour was observed (Nyasembe *et al.*, 2012). In choice tests presenting several synthetic lemon leaf volatiles, the Fuller's rose weevil (*Pantomorus cervinus* Boheman; Coleoptera: Curculionidae) showed a positive response to medium dosages of the treatments tested (50.0 mg/ 100 mL), while no significant differences were observed between the treatments and solvent controls at the two lower concentrations (0.5 and 5.0 mg/ 100 mL). Additionally, strong repellence to several synthetic lemon leaf volatiles was noted at the highest concentration (500.0 mg/ 100 mL) (Wee *et al.*, 2008). In this study, the dose of volatiles emitted by apple sauce had also an effect on vine weevil behaviour, with adults showing a preference for the apple odour stimuli presented at lower and medium (0.1 g, 1 g and 10 g) doses tested compared to the highest (20 g) when compared to the controls. It is known that insects rely on quantitative variations in volatile compounds to identify and differentiate between host plants. As a result, further research is required to examine the concentrations of individual active components in these blends, in order to create effective fruit-based lures for managing vine weevils.

To enhance knowledge of the interaction of behaviourally effective constituents in complex odour blends and contribute the development of an efficient monitoring system involving olfactory cues this study investigated also the effect of apple sauce when presented alongside or in the absence of other host volatiles such as spindle. When presented a choice between the spindle host plant and apple sauce no significant differences were found in both laboratory and glasshouse tests. However, the monitoring tool baited with spindle plus apple recorded the highest number of weevils compared to those completely empty or only baited with apple or spindle.

Bruce and Pickett (2011) noted that when a compound is tested individually outside the context of the blend, insects can perceive this compound as non-host cues but when compounds are combined together in a blend they are perceived as a host stimulus. This response to specific blends of plant volatiles to recognise hosts (Bruce and Pickett, 2011) has been reported also for other weevil species (Collatz and Dorn, 2013; Piesik and Wenda-Piesik, 2015) and it is known to increase monitoring tool efficacy. For example, a six-compound blend (6-methyl-5-hepten-2-one, (*E*)- $\beta$ -caryophyllene, 1-octanol, benzaldehyde, benzyl alcohol, benzonitrile and decane) significantly increased the number of captured apple blossom weevil

(*Anthonomus pomorum* L.; Coleoptera: Curculionidae) individuals (Collatz and Dorn, 2013). Traps baited with a combination of 2-hexenal and benzaldehyde were found to increase the number of pulse beetle (*Callosobruchus chinensis* L.; Coleoptera: Bruchidae) individuals compared to unbaited traps in crop fields (Wang *et al.*, 2020). Although vine weevil responses to blends tested here and in previous studies (Roberts *et al.*, 2019b) have been shown to elicit positive behavioural responses, further studies are needed to identify the odour mixtures that have the greatest effect on vine weevil behaviour to further improve monitoring tool efficacy.

Several studies have reported the use of monitoring tools baited with natural lures (e.g., food-based) or fermented products, vinegars, wines, yeast-sugar solutions and fruit juices to improve monitoring tool efficacy (Cha *et al.*, 2013). For example, cut orange peels and orange juice have been reported to increase catches of the Mediterranean fruit fly (*Ceratitis capitata* Wiedemann; Diptera: Tephritidae), but differences in effectiveness were observed when different tissues and preparations were tested (Katsoyannos *et al.*, 1997). These natural baits have been widely recommended (e.g., for the control of Tephritidae and Drosophilidae spp. as well as stored-product insects) for their ubiquity, simplicity and cost effectiveness (Rosa *et al.*, 2017). Despite this, one of the main disadvantages of these lures is variability due, for example, to a lack of standardisation of industrial manufacture (Rosa *et al.*, 2017). Low efficacy over the time, sex-biased captures, the need for frequent re-baiting have been reported to reduce monitoring tool efficacy (Candia *et al.*, 2019). Furthermore, these food lures have been shown to increase catches of a broad range of non-target and beneficial insects (Cha *et al.*, 2013) including predatory wasps, bees and lacewings (Candia *et al.*, 2019). For these reasons, although the commercial product based on apple sauce may play an important role in the vine weevil monitoring, further studies are needed to identify key bioactive compounds originating from apple volatiles to develop synthetic lures which would improve vine weevil monitoring tool efficacy.

Apple volatiles, in particular  $\alpha$ -farnesene (a bioactive volatile released from the wax of apple skin), have been shown to influence the behaviour of mated female codling moths (*Cydia pomonella* L.; Lepidoptera: Tortricidae), stimulating oviposition (Wearing and Hutchins, 1973; Sutherland *et al.*, 1977). Similarly, Lin *et al.* (2015) found that the food-odour apple cider vinegar trigger the fruit fly (*Drosophila melanogaster* Meigen; Diptera: Drosophilidae) males to release the pheromone 9-tricosene. This pheromone acts as a potent aggregation pheromone as well as a chemosensory cue to influence female oviposition site selection. Hedin and Mccarty (1990) showed that the sugars (glucose, fructose, and sucrose) found in the cotton (*Gossypium* spp.; Malvales: Malvaceae) anthers stimulated oviposition in the boll weevil (*Anthonomus grandis* Boheman; Coleoptera: Curculionidae) females. In this study, during the experiments carried out under glasshouse conditions, a significant proportion of vine weevil eggs was found in monitoring tool baited with apple sauce compared to unbaited or baited

with other olfactory stimuli (personal communication). Little is known about the effect of volatiles released from the apple fruit on vine weevil behaviour. Therefore, more work is required to further understand the role that these apple-based stimuli may play in host finding and oviposition by vine weevil.

This study provides insights into the potential use of apple sauce volatiles as attractants for the development of semiochemical-based IPM approaches for the control of vine weevil. Results showed that apple sauce elicit positive behavioural responses in vine weevil adults under both laboratory and glasshouse settings. Similarly, higher numbers of vine weevil adults were recorded in monitoring tools with apple and spindle than in monitoring tool baited with only apple or spindle. However, the specific volatiles responsible for these responses have yet to be identified. Future investigations will need to focus on identifying these volatiles, as understanding the precise chemical cues is crucial for optimising lures and enhancing the efficacy of semiochemical-based IPM approaches. Much remains to be studied also in the field of interactions between vine weevil, apple-based lures and environmental factors such as temperature and humidity, but it believed that apple-based lures may play an important role in vine weevil IPM programme.

# Chapter 5: Investigating the Effect of a Garlic-Based Product, Pitcher GR<sup>®</sup>, on Vine Weevil and Its Compatibility with Entomopathogenic Nematodes<sup>4</sup>

## Abstract

Plant protection products derived from plant material are a sustainable alternative to conventional synthetic chemical pesticides. This study investigates the efficacy of a commercially available bioinsecticide based on garlic (*Allium sativum* L.; Asparagales: Amaryllidaceae) extract against vine weevil eggs and larvae under laboratory and glasshouse conditions. In addition, the compatibility of this product with the entomopathogenic nematode, *Steinernema kraussei* (Steiner; Rhabditida: Steinernematidae), was investigated under laboratory and glasshouse conditions. Results showed that garlic significantly reduced egg hatch when applied as fumigant or as combined of fumigant and direct contact. No effect was observed when the garlic product was applied as a contact application only. This garlic-based bioinsecticide also significantly reduced larval survival when either contact or fumigant applications were used. A combined contact and fumigant effect was shown also when vine weevil eggs were exposed to the bioinsecticide for 30 days in plastic containers containing growing media. The number of larvae recovered after this period was significantly reduced compared to the control group. However, despite these positive results, Pitcher GR<sup>®</sup> reduced the survival of *S. kraussei* under laboratory conditions. When the bioinsecticide was tested under glasshouse conditions, the number of larvae recovered from Primula or Begonia potted plants did not differ from that of the control group. Similarly, the combination of Pitcher GR<sup>®</sup> and *S. kraussei* was only effective when the two controls were applied 27 days apart. However, application of *S. kraussei* alone was effective in reducing the number of larvae recovered from potted Begonia plants compared to the control group.

This study provides a first step toward understanding the potential of using garlic-based bioinsecticides, such as Pitcher GR<sup>®</sup>, for vine weevil control. Further studies are, however, needed to determine whether such bioinsecticides can be used effectively under field conditions.

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<sup>4</sup> Sections of the following Chapter have been published in:

1: Fezza *et al.* (2024). The Garlic Gambit: an alternative strategy for controlling vine weevil (*Otiorynchus sulcatus* F.; Coleoptera: Curculionidae). Journal of Economic Entomology, XX(XX), 2024, 1–11 <https://doi.org/10.1093/jee/toae175>.

## 1. Introduction

Insect pest management has relied on the use of synthetic chemical insecticides, such as carbamates, organophosphates, pyrethroids and neonicotinoids over the past six decades (Chandler *et al.*, 2011; Sparks *et al.*, 2021). Whilst these products have proven to be effective, alternative and more sustainable control measures are required to avoid environmental contamination, reduce risk to non-target organisms and to combat the increasing number of insecticide resistant pest populations (Barzman *et al.*, 2015; Chaudhary *et al.*, 2017). One such alternative is the use of botanical bioinsecticides formulated using naturally occurring secondary metabolites (e.g., fatty acids, alkaloids, terpenoids, phenols, quinones, tannins and flavonoids) extracted from plant material (roots, leaves, stems, fruits, etc.) (Pavela, 2016). These phytochemicals have been shown to be effective for controlling insect pests by disrupting key physiological processes, such as moulting and respiration as well as exciting the central nervous system (Pavela and Benelli, 2016; Lengai *et al.*, 2020). Phytochemicals have also been shown to modify insect behaviour by acting as attractants, deterrents, phago-stimulants, antifeedants or anti-oviposition cues (Isman, 2006). The bioactivity of many phytochemicals on a wide range of insect pests is complemented by their low cost, lack of residual effects, availability and compatibility with circular economy principles (Isman, 2006). Due to their chemical complexity, phytochemicals are also less susceptible to pest populations developing resistance as there may be several modes of action (Lengai *et al.*, 2020). Botanical bioinsecticides, therefore, represent a useful tool that could be incorporated into integrated pest management (IPM) programmes alongside conventional synthetic insecticides (Isman *et al.*, 2011) and biological control agents (Benelli *et al.*, 2018).

Garlic (*Allium sativum* L.; Asparagales: Amaryllidaceae) is a perennial species of bulbous flowering plant closely related to onions, leeks and chives, that is native to Asia (Block, 2009). This plant species is widely cultivated for culinary use as a seasoning due to its distinct flavour profile resulting from high levels of bioactive phytochemicals found in the bulb, including allicin (2-propene-1-sulfinothioic acid S-2-propenyl ester), diallyl sulfide (3-prop-2-enylsulfanylprop-1-ene), diallyl disulfide (3-(prop-2-enylsulfanyl)prop-1-ene) and diallyl trisulfide (3-(prop-2-enyltrisulfanyl)prop-1-ene) (Huang *et al.*, 2000).

Garlic extracts (e.g., oil and powder) have been shown to possess insecticidal, repellent, anti-feeding and anti-microbial properties (Mamduh *et al.*, 2017). Furthermore, garlic extracts applied as fumigant and contact applications have been reported to induce lethal and sublethal effects to several stored product pest species, including the rice weevil (*Sitophilus oryzae* L.; Coleoptera: Curculionidae), the maize weevil (*S. zeamais* Motschulsky; Coleoptera: Curculionidae) and the red flour beetle (*Tribolium castaneum* Herbst; Coleoptera: Tenebrionidae) (Ho *et al.*, 1996; Huang *et al.*, 2000). In contact bioassays, for example, garlic

essential oil has been reported to induce symptoms such as necrosis in mealworm beetle (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae) larvae, pupae and adults twenty-four hours after exposure (Plata-Rueda *et al.*, 2017). Other effects of garlic extracts include decreased hatching rates and embryonic development (Mamduh *et al.*, 2017). Diallyl trisulfide, an active substance from garlic essential oil, has been shown to significantly downregulate the mRNA of chitin synthase A (CHSA) gene (a conserved enzyme responsible for the completion of chitin biosynthetic pathway) in the Angoumois grain moth (*Sitotroga cerealella* Olivier; Lepidoptera: Gelechiidae) (Shah *et al.*, 2020). Downregulation of CHSA resulted in chitin reduction, epidermal disruption, morphological and physiological alterations as well as oviposition reduction (Shah *et al.*, 2020). Currently, there are several products based on garlic extracts (e.g., Mosquito Barrier<sup>®</sup>; Chinese garlic oil) and these are marketed (e.g., in the United States and Canada) as bioinsecticides and repellents for the management of many insect pests (Dusi *et al.*, 2022).

Recent studies have shown that combining two different management strategies can result in improving pest suppression than when used individually (Reddy and Chowdary, 2021). Botanical bioinsecticides are often used in conjunction with other IPM strategies such as entomopathogenic nematodes (EPNs) or natural enemies (Amoabeng *et al.*, 2019; Reddy and Chowdary, 2021). This reduces the cost of biological control applications as well as grower reliance on synthetic chemical insecticides (Guo *et al.*, 2016). Initial research has shown that plant-based products may not affect the survival, virulence or infectivity of EPNs and natural enemies when combined. For example, Laznic and Trdan (2014) reported the compatibility of azadirachtin with *Steinernema feltiae* (Filipjev; Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Poinar; Rhabditida: Heterorhabditidae).

This study evaluated the potential role of a granular garlic-extract bioinsecticide, Pitcher GR<sup>®</sup>, commercially available in the UK, as a management option for use against vine weevil eggs and larvae under laboratory and glasshouse conditions. In addition, the compatibility of Pitcher GR<sup>®</sup> with the EPN species, *S. kraussei*, as well as any benefits from combined applications of Pitcher GR<sup>®</sup> with the same species of EPN was assessed under laboratory and glasshouse conditions.

It is hypothesised that this commercially available bioinsecticide based on garlic would: (i) affect the survival of vine egg and larva under both laboratory and glasshouse conditions and (ii) not affect the survival of the entomopathogenic nematode (*S. kraussei*) when applied simultaneously.

## **2. Materials and Methods**

### **2.1 Insect Cultures**

Adult vine weevils were collected from commercial strawberry (*Fragaria x ananassa* cv. *Duchesne*) crops in Staffordshire (UK) during summer 2022. Thirty adults were placed into four terraria (30 × 19.3 × 20.6 cm; Exo Terra, Castleford, UK) containing yew (*Taxus baccata* L.; Cupressales: Taxaceae) sprigs with moist paper towels as a food/water source and cotton wool balls to act as an oviposition site. Yew, paper towels and cotton wool balls were replaced weekly. These terraria were housed in a controlled environment room maintained at 20 °C and 60 % relative humidity with a 16:8 light:dark photoperiod (Fitotron, Weiss Technik, Ebbw Vale, Wales, UK).

### **2.2 Begonia and Primula plants**

Wax begonia (*Begonia x semperflorens-cultorum*, Hort; Cucurbitales: Begoniaceae) and common primrose (*Primula vulgaris* Huds; Ericales: Primulaceae) plants were purchased from a local garden centre (Mere Park Garden Centre, Newport, UK). Purchased plants were transplanted into new growing media (SylvaGrow® John Innes Number 2, Tetbury, UK) inside 1L pots (13 cm diameter; Teku VCH13, Pöppelmann, Lohne, Germany) and grown for four weeks under glasshouse conditions (Begonia plants: ≈ 21° C; 66 % relative humidity; Primula plants: ≈ 24° C; 42 % relative humidity) before using for experiments. Plants were watered every two to three days as required, by pouring water into plastic saucers (16 cm in diameter; Whitefurze Ltd, UK).

### **2.3 Entomopathogenic nematodes solution**

Nemasys® (*Steinernema kraussei*, BASF, Stockport, UK) nematodes in packs of 6 million were used for both laboratory experiments and glasshouse experiments. Nematodes were stored at 5.0 °C (Lec LSR151, Merseyside, UK). To prepare the nematode stock solution, a single pack of nematodes was diluted in tap water (e.g., 1.2 Liters for packs containing 6 million nematodes) and aerated for five minutes using a Tetra APS50 air pump (Malle, Germany). A viability count was then conducted by taking 1 ml of this solution, diluting it with tap water up to 200 ml in a plastic jug (Firtink, UK). Then 1 ml of this dilution was pipetted into a 3 × 5 cm plastic counting chamber with gridlines and live infective juveniles (IJs) were counted under a dissecting microscope (Microtec HM3, Somerset, UK). This process was

repeated in triplicate and the entire procedure was performed three times (for a total of nine counts) to estimate the number of live IJs in the stock solution. Based on this, the desired concentrations were created to complete laboratory and glasshouse bioassays.

## 2.4 Bioinsecticide Treatment

Pitcher GR<sup>®</sup> (ICL Specialty Fertilizers, UK) is a granular bioinsecticide based on garlic extract (active ingredient: 450 g / kg garlic extract) commercially available in the United Kingdom (approved through an Extension of Authorisation for Minor Use; Ministerially Approved Pesticide Product (MAPP) Number: 18126) for the control of vine weevil eggs and young larvae in containerised ornamental plants (ICL Specialty Fertilizers, 2024). The recommended application rate is 240 kg per ha (24 g per m<sup>2</sup>). Based on this, 0.42 g or 0.06 g used in the laboratory experiments were calculated for the area of plastic containers (170 cm<sup>2</sup>) and Petri dishes (28.26 cm<sup>2</sup>), respectively (Table 1). Whereas 0.038 g (area of plastic pot = 15.89 cm<sup>2</sup>) and 0.32 g (area of plant pot = 132.73 cm<sup>2</sup>) were used for the experiments investigating the effect of Pitcher GR<sup>®</sup> on the EPN, *S. kraussei* (Table 1).

**Table 1.** Summary of each application rate of Pitcher GR<sup>®</sup> used for the bioassays carried out under laboratory and glasshouse conditions.

Type of bioassay	Experimental unit	Experimental unit area (cm <sup>2</sup> )	Application rate of Pitcher GR <sup>®</sup> (g)
Toxicity screening on vine weevil eggs	Plastic container	170	0.42
Toxicity screening on vine weevil larvae	Petri dishes	28.26	0.06
Compatibility Bioassay	Plastic pot	15.89	0.038
Efficacy Bioassay	Begonia pot	132.73	0.32
Toxicity screening on vine weevil eggs/larvae	Primula pot	132.73	0.32

## 2.5 Toxicity Screening on Vine Weevil Eggs

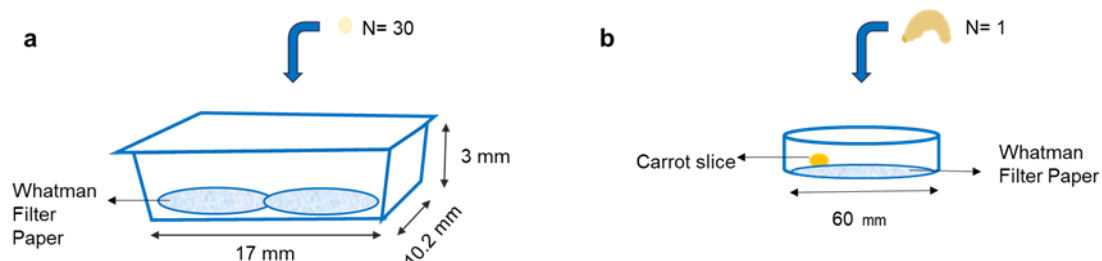
### 2.5.1 Egg Collection

Eggs were collected every three days from the cotton wool balls in each terrarium using a fine 000 paintbrush. All eggs were examined under a binocular microscope (Microtec HM3) to ensure that only undamaged, fully melanised eggs were used for an experimental batch.

These experimental batches (approximately 100 eggs) were then stored at 5 °C (Lec LSR151, Merseyside, UK) in plastic containers (115 x 40 mm, Signature Packaging, UK) until they were used in a bioassay. Eggs were used in bioassays within seven days of collection from the terraria. Egg viability counts were carried out for each experiment by maintaining twenty eggs on moist tissue paper in a 90 mm diameter plastic Petri dish (Thermo Scientific™ 101VR20, Fisher Scientific UK Ltd) in a controlled environment room (Fitotron) at 20 °C and 60 % relative humidity in complete darkness. The remaining egg batches were maintained under the same environmental conditions in plastic containers (17 x 10.2 x 3 cm, GP Globe Packaging, UK) filled with compost (SylvaGrow® John Innes Number 2) within a controlled environment room under the same conditions previously described (Fitotron) to produce larvae for toxicity screening.

### 2.5.2 Bioassay Experimental Units

Laboratory bioassays were carried out to determine the toxicity of Pitcher GR® through contact and fumigation as well as combined contact and fumigation on vine weevil eggs. Experimental units consisted of plastic containers (17 x 10.2 x 3 cm, Area = 170 cm<sup>2</sup>; GP Globe Packaging, UK) (Fig. 1a) lined with two filter papers (Ø = 110 mm; Fisherbrand™, Fisher Scientific UK Ltd). Thirty vine weevil eggs were then transferred to each container using a fine 000 paintbrush. Experimental units were then placed in a controlled environment room maintained at 20 °C and 60 % relative humidity (Fitotron) under complete darkness. Eggs were monitored daily for thirty days and at the end of this period, egg viability was assessed by counting the total number of hatched eggs. Eggs were considered dead when they became opaque, necrotic and/or appeared dehydrated.



**Figure 1.** Schematic diagram showing the experimental set-up used to determine the toxic effect of a garlic-based product on vine weevil eggs (a) and larvae (b).

### **2.5.3 Contact Toxicity Bioassay**

Contact toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs was determined in a direct contact bioassay. This was achieved by using the experimental units previously described in 2.5.2 Bioassay Experimental Units Section. In the control experimental units 3 ml of water was pipetted into filter papers while 0.42 g of Pitcher GR<sup>®</sup> and 3 ml of water was added to the filter papers in the treatment experimental units. Adding water to the experimental units ensured that the eggs were hydrated and the filter papers were re-wetted with 1 ml of water as required. Experimental units were left unsealed in this bioassay to minimise the potential fumigant effect of Pitcher GR<sup>®</sup>. Ten replicate experimental units were prepared for each control and treatment with a total of 600 eggs tested (300 in the treated group and 300 in the untreated group).

### **2.5.4 Fumigant Toxicity Bioassay**

Fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs was determined in a non-contact fumigation bioassay. This was achieved by using the experimental units previously described for the 2.5.3 Contact Toxicity Bioassay but adding Petri dishes (35 x 11 mm; Fisher Scientific, UK Ltd) containing either 3 ml of water (control experimental units) or 3 ml of water and 0.42 g of Pitcher GR<sup>®</sup> granules (treatment experimental units) to prevent direct contact between the vine weevil eggs and Pitcher GR<sup>®</sup> granules. Experimental units were sealed with airtight lids. All the experimental units initially received 3 ml of water to their filter papers to ensure that eggs were sufficiently hydrated and re-wetted with 1 ml of water as required. Ten replicate experimental units were prepared for each control and treatment with a total of 600 eggs tested (300 in the treated group and 300 in the untreated group).

### **2.5.5 Combined Contact and Fumigant Toxicity Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs was determined in a contact fumigation bioassay. This was achieved using the bioassay set up described for the 2.5.3 Contact Toxicity Bioassay but also sealing the experimental units with airtight lids. In the control experimental units 3 ml of water was pipetted onto filter papers while 0.42 g of Pitcher GR<sup>®</sup> and 3 ml of water was added to the filter papers in the treatment experimental units. All experimental units initially received 3 ml of water to their filter papers to ensure that eggs were sufficiently hydrated and re-wetted with 1 ml of water as required. Ten replicate experimental units were prepared for each control and treatment with a total of 600 eggs tested (300 in the treated group and 300 in the untreated group).

## **2.5.6 Dose Response Toxicity Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs at varying concentrations was determined in a contact fumigant bioassay. This was achieved using the bioassay set up described in Section 2.5.3, for the Contact Toxicity Bioassay but also sealing the experimental units with airtight lids. Pitcher GR<sup>®</sup> was applied at four different concentrations 0.1%, 1%, 10% and 100 % which gave a dosage of 0.0004 g, 0.004 g, 0.04 g, 0.4 g respectively. The control experimental units received only 3 ml of water pipetted onto filter papers. All experimental units initially received 3 ml of water to their filter papers to ensure eggs were sufficiently hydrated and re-wetted with 1 ml of water as required. Five replicate experimental units were prepared per each control and treatment dose with a total of 750 eggs tested (150 per each treatment).

## **2.6 Toxicity Screening on Vine Weevil Larvae**

### **2.6.1 Larva production**

Vine weevil eggs collected as described in the Egg Collection Section were maintained in plastic containers (17 x 10.2 x 3 cm, GP Globe Packaging, UK) filled with growing media (SylvaGrow<sup>®</sup> John Innes Number 2). Carrot slices were placed on the surface of the growing media each week in plastic containers as a food source for emerging larvae. These containers were housed in a controlled environment room at 20 °C and 60 % relative humidity (Fitotron) under complete darkness to produce larvae for toxicity screening.

### **2.6.2 Bioassay Experimental Units**

Laboratory bioassays were carried out to determine the toxicity of Pitcher GR<sup>®</sup> through contact or fumigation as well as combined contact and fumigation on vine weevil larvae. Experimental units consisted of plastic Petri dishes (60 x 15 mm; Area = 28.26 cm<sup>2</sup>; Sarstedt AG & Co. KG, Germany) (Fig. 1 b) lined with filter papers (Ø = 47 mm; Whatman<sup>™</sup>, Fisher Scientific UK Ltd). Individual vine weevil larvae (first-instar larvae, max 20 days old; body length ≈ 3.2 mm, head capsule width ≈ 0.62 mm) were added to each Petri dish using a 000 fine paintbrush. Once the treatments being tested were added to these experimental units, they were maintained under complete darkness in a controlled environment cabinet (Panasonic, MLR- 352- PE, Japan) set to 20 °C. A carrot slice (Ø = 1 cm) was placed in each experimental unit as a food source. Larvae were monitored daily for seven days and after this

period the number of surviving individuals was recorded. Larval mortality was determined by gently stimulating individuals with a 000 fine paintbrush and observing movement, with no movement indicating mortality.

### **2.6.3 Contact Toxicity Bioassay**

Contact toxicity of Pitcher GR<sup>®</sup> to vine weevil larva was determined in a direct contact bioassay. This was achieved by using the experimental units previously described in the 2.6.2 Bioassay Experimental Units Section. In the control experimental units 2 ml of water was pipetted into filter papers while 0.06 g of Pitcher GR<sup>®</sup> and 2 ml of water was added to the filter papers in the treatment experimental units. Adding water to the experimental units ensured that larvae were hydrated, and the filter papers were re-wetted with 1 ml of water as required throughout the bioassay. Experimental units were left unsealed in this bioassay to eliminate the potential fumigant effect of Pitcher GR<sup>®</sup>. Thirty replicate experimental units were prepared for each control and treatment with a total of 60 larvae tested (30 in the control group and 30 in the treatment group).

### **2.6.4 Fumigant Toxicity Bioassay**

Fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil larvae was determined in a non-contact fumigation bioassay. This was achieved by using the experimental units previously described for the 2.6.3 Contact Toxicity Bioassay but attaching a smaller Petri dish (35 x 10 mm; Sarstedt AG & Co. KG, Germany) containing 2 ml of water (control experimental units) or 2 ml of water and 0.06 g of Pitcher GR<sup>®</sup> granules (treatment experimental units) to the larger Petri dish with glue. All the experimental units were sealed with airtight lids. A drill (Dremel 3000, Eternal Tools, UK) was used to make a 1 cm hole in the larger Petri dishes to allow volatiles from the treatment to enter the experimental unit. Fine mesh was glued over this hole to prevent direct contact between the larvae and the treatments. Adding water to the experimental units ensured that larvae were hydrated, and the filter papers were re-wetted with 1 ml of water as required throughout the bioassay. Thirty replicate experimental units were prepared for each control and treatment with a total of 60 larvae tested (30 in the control group and 30 in the treatment group).

### **2.6.5 Combined Contact and Fumigant Toxicity Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil larvae was determined in a contact fumigant bioassay. This was achieved using the bioassay set up described for the 2.6.3 Contact Toxicity Bioassay but also sealing the experimental units with airtight lids. In the control experimental units 2 ml of water was pipetted into filter papers while 0.06 g of Pitcher GR<sup>®</sup> and 2 ml of water was added to the filter papers in the treatment experimental units. Adding water to the experimental units ensured that larvae were hydrated, and the filter papers were re-wetted with 1 ml of water as required throughout the bioassay. Thirty replicate experimental units were prepared for each control and treatment with a total of 60 larvae tested (30 in the control group and 30 in the treatment group).

### **2.7 Growing Media Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs under semi-field conditions was determined in a contact and fumigant bioassay. This was achieved by taking the experimental units (e.g., plastic containers) described in the 2.5.2 Bioassay Experimental Units Section and filling with 100 g of growing media (SylvaGrow<sup>®</sup> John Innes Number 2) before adding thirty vine weevil eggs onto the growing media surface using a 000 fine paintbrush then sealing with airtight lids. In the control experimental units 3 ml of water was added while 0.42 g of Pitcher GR<sup>®</sup> and 3 ml of water was added in the treatment experimental units. All experimental units initially received 3 ml of water to ensure that eggs were sufficiently hydrated and re-wetted with 1 ml of water as required. Carrot slices were placed in each experimental unit as a food source for emerging larvae. Experimental units were assessed after 30 days with the number of larvae recorded per unit. Ten replicate experimental units were prepared for each control and treatment with a total of 600 eggs tested (300 in the treated group and 300 in the untreated group).

### **2.8 Compatibility Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to the EPN species, *S. kraussei*, under semi-field conditions was determined in a contact and fumigant bioassay. Experimental units (control and treatment) consisted of 50 ml plastic pots (Fabri-Kal GPC200, Illinois, USA), each filled with 20 ml of growing media (SylvaGrow<sup>®</sup> John Innes Number 2) (Shapiro-Ilan, Stuart and McCoy, 2006). To set the moisture levels, 200 g of the growing media was submerged in tap water for 30 minutes in 1 L glass jars (Teku VCH 13, Pöppelmann,

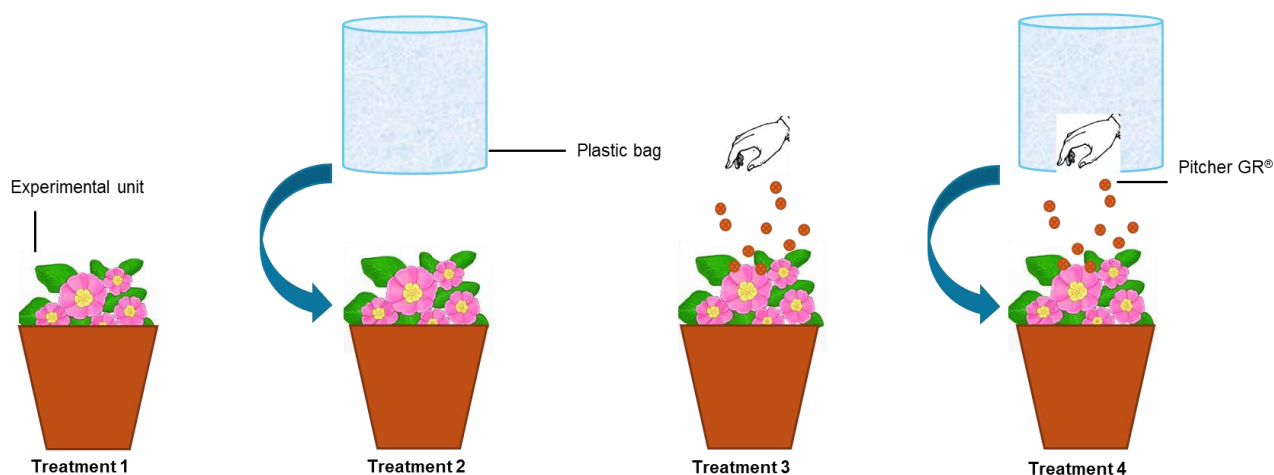
Lohne, Germany). Then the growing media was removed and drained for one hour in organza bags (13 × 18 cm; Amison, UK) until no more water dripped, achieving full saturation (Hartley and Wallace, 2017). The experimental units were weighed at the beginning of the experiment, and each week the pots were opened to allow air exchange and to add water, returning each unit to its initial weight (Koppenhöfer and Fuzy, 2007). Each experimental unit was inoculated with 500µl of the stock solution (20,000 IJs), described previously in Section 2.3. Pitcher GR<sup>®</sup> (0.038 g) was added to the treatment experimental units one hour after inoculation with EPNs, whereas in the control experimental units only 500µl of tap water was applied. Experimental units were then placed in a controlled environment room (20 °C; 60 % relative humidity; Fitotron) and maintained in the dark for the entire duration of the experiments. One day after inoculation, the number of live EPNs was assessed by washing the entire contents of a pot in 300 ml of tap water. From this solution, 1 ml was pipetted into a 3 × 5 cm counting chamber with gridlines and live IJs were counted under a dissecting microscope (Microtec HM3, Somerset, UK). This procedure was performed in triplicate to estimate the total number of live EPNs in each pot. The process was repeated on days 1, 3, 7, 14, and 21 after nematodes were applied to each pot. The whole experiment was repeated five times.

## **2.9 Glasshouse Experiments**

### **2.9.1 Combined Contact and Fumigant Toxicity Bioassay**

Combined contact and fumigant toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs under glasshouse conditions was determined in a contact and fumigant bioassay. This was achieved by using experimental units consisting of *Primula* plants grown in 13 cm diameter pots (Teku VCH13, Pöppelmann, Lohne, Germany), previously described in Section 2.2. For each experimental units (controls and treatments) twenty vine weevil eggs (previously described in Section 2.4.1) were placed around the plant stem using a 000 fine paintbrush (Meeden<sup>®</sup>, China). After 1 hour, in the control experimental units 50 ml of water was applied around the plant stem of each plant while 0.32 g of Pitcher GR<sup>®</sup> and 50 ml of water was applied in the treatment experimental units. Experimental units were covered, as required, with plastic bags (20 x 28 cm, Sabco, UK) (Fig. 2) in this bioassay to maximise the potential fumigant effect of Pitcher GR<sup>®</sup> so that four treatments were created: 1) control experimental units covered with bags; 2) control experimental units not covered with bag; 3) treatment experimental units covered with bags and 4) treatment experimental units not covered with bags. Experimental units (controls and treatments) were arranged in a randomised block design. Ten experimental units were used per each treatment (Fig. 2), including for the control experimental units. A

viability count of vine weevil eggs was carried out by keeping twenty eggs on moist tissue paper in a 90 mm diameter Petri-dish as previously described. After 60 days each *Primula* plant was checked and the number of live larvae in each pot was recorded. Glasshouse temperatures were recorded with SensorPush HT1Smart Sensors (New York, USA). SensorPush HT.w Water-Resistant Smart Sensors (New York, USA) were set up in an experimental unit to record temperature and humidity (Table 2).



**Figure 2.** Schematic diagram showing the experimental set-up used to determine the efficacy of garlic-based product on vine weevil eggs under semi-field conditions.

**Table 2.** Temperature and humidity (means  $\pm$  S.E.) recorded in the Combined Contact and Fumigant Toxicity Bioassay under glasshouse conditions.

No. Treatment	Temperature ( $^{\circ}$ C)	Humidity (%)
1 & 3	23.75 $\pm$ 0.7	41.74 $\pm$ 1.14
2 & 4	26.06 $\pm$ 0.4	87.85 $\pm$ 0.3

Temperature and Humidity were recorded with SensorPush HT.w Water-Resistant Smart Sensors set up close *Primula* arial parts.

### 2.9.2 Efficacy Bioassay

A glasshouse experiment was carried out to evaluate efficacy of Pitcher GR<sup>®</sup> and/or the EPN species, *S. krausseii*, against vine weevil eggs and larvae. Experimental units consisted of *Begonia* potted plants (13 cm diameter; Teku VCH13)(Section 2.2) with seven

treatments (Table 3). Each required experimental unit was infested with vine weevil eggs (Table 3), collected from the terraria containing vine weevil adults as previously described in Section 2.5.1. After twenty-four hours or twenty-eight days the vine weevil eggs were applied, 100 ml (Section 2.3) of the nematode dilution (with 25000 IJs) was poured in a plastic bottle fitted with a sprinkler (15 x 10 x 2 cm; SuonaOmni) and applied to each required EPN-treated experimental unit. In the treatment experimental units this solution was sprayed directly onto the surface of the growing media while 100 ml of water was sprayed directly onto the surface of the growing media of the control experimental units (e.g., treatments 0 and 1; Table 3). Similarly, in the required treatment experimental units 0.32 g of Pitcher GR<sup>®</sup> and 50 ml of water was applied around the plant stem of each plant while 50 ml of water was applied in the other experimental units. Plants were watered every 2-3 days as required, by pouring water into individual plastic saucers (16 cm in diameter; Whitefurze Ltd, UK) to prevent EPN contamination between treatments and so as not to wash out the EPNs. A viability count was carried out by keeping twenty eggs on moist tissue paper in a 90 mm diameter Petri-dish under laboratory conditions (20 °C; 60 % relative humidity; 24 hours of darkness). Experimental units were arranged in a randomised block design. Ten replicates per each treatment were prepared, including for the control treatments (treatment 0 and 1; Table 3), with a total of 70 Begonia plants tested. The experiment ran for fifty-six days from the application of vine weevil eggs. After this period each Begonia potted plant was checked and the number of surviving larvae in each pot was recorded.

**Table 3.** Treatments used for the Efficacy Bioassay using Pitcher GR<sup>®</sup> and the entomopathogenic nematodes, *S. kraussei*, carried out under semi-field conditions.

Treatment	Tested Combination of EPN and/or Pitcher GR <sup>®</sup>	Abbreviations	No. of Replicates
0	Begonia with no eggs no Pitcher no EPNs		10
1	Begonia with eggs <sup>a</sup> no Pitcher no EPNs	Control	10
2	Begonia with eggs and Pitcher <sup>b</sup> (applied 24hrs after the eggs were applied) no EPNs	P24	10
3	Begonia with eggs and Pitcher and EPNs <sup>c</sup> (applied 24hrs after the eggs were applied)	P24SK24	10
4	Begonia with eggs and Pitcher and EPNs (pitcher applied 24hrs after eggs applied and EPNs applied 28 days after eggs were applied)	P24SK28	10
5	Begonia with eggs and Pitcher and EPNs (applied 28 days after the eggs were applied)	P28SK28	10
6	Begonia with eggs and EPNs (applied 28 days after the eggs were applied) no Pitcher	SK28	10

<sup>a</sup>20 vine weevil eggs per each Begonia plants were used for this Bioassay.

<sup>b</sup> 0.32 g of Pitcher GR<sup>®</sup> were applied to each Begonia plants.

<sup>c</sup> A solution of 100ml containing 25000 IJs of *S. kraussei* was applied to each required EPN-treated potted plant .

### 3. Statistical Analysis

Statistical analyses were carried out using R v4.3.2 (R Core Team, 2022). In bioassays testing the toxicity of Pitcher GR<sup>®</sup> to vine weevil eggs through different exposures routes, count data were analysed using generalised linear models (GLMs) fitted to a Poisson or quasi-Poisson probability distribution depending on the presence of overdispersion. This was determined using `qcc.overdispersion.test` function from the `qcc` package (Scrucca, 2004), where a p-value greater than 0.05 indicated no overdispersion and justifies the use of Poisson distribution. In bioassays testing the toxicity of Pitcher GR<sup>®</sup> to vine weevil larvae count data for larvae (Section 2.6) were analysed with GLMs fitted to a binomial probability distribution as there were only two outcomes in the bioassays: live or dead. In Dose Response Toxicity Bioassay (Section 2.5.5) and Growing Media Bioassay (Section 2.7) count data for eggs and larvae were analysed using generalised linear models (GLMs) fitted to a quasi-Poisson probability distribution to account for overdispersion. Similarly, to analyse the mean of larvae/nematodes recovered in the bioassays (e.g., Section 2.8, 2.9.1 and 2.9.2) count data were analysed using GLMs fitted to a quasi-Poisson probability distribution after testing for overdispersion as previously described. Multiple comparisons for quasi-Poisson probability distribution data were evaluated by Tukey's HSD tests implemented in the `HSD.test` function in the `agricolae` R package (De Mendiburu, 2019).

## 4. Results

### 4.1 Vine Weevil Eggs

#### 4.1.1 Contact Toxicity Bioassay

Exposing vine weevil eggs to Pitcher GR<sup>®</sup> in a direct contact toxicity bioassay had no effect on egg hatch rate compared to those exposed to water (GLM with a Poisson distribution:  $z = -1.069$ ,  $df = 18$ ,  $p = 0.285$ ). The mean percentage of hatched eggs for the control treatments was  $95 \pm 1.3$  % while for Pitcher GR<sup>®</sup> treatment the mean percentage of hatched eggs was  $87.0 \pm 2.3$  % (Fig. 3a).

#### 4.1.2 Fumigant Toxicity Bioassay

Exposing vine weevil eggs to Pitcher GR<sup>®</sup> in a fumigation toxicity bioassay significantly reduced egg hatch rate compared to those exposed to water (GLM with a quasi-Poisson

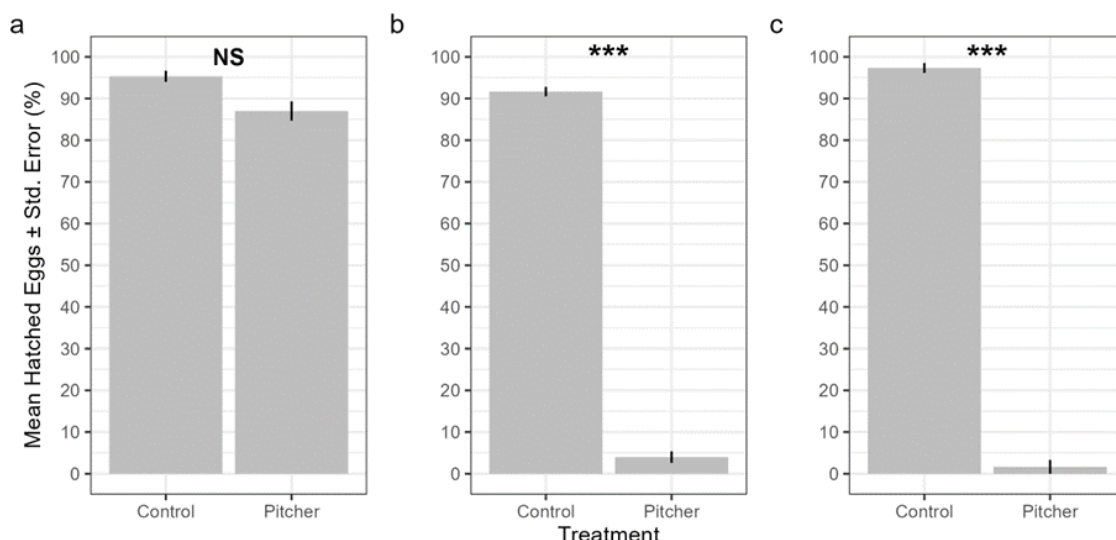
distribution:  $t = -12.32$ ,  $df = 18$ ,  $p < 0.001$ ). The mean percentage of hatched eggs decreased from  $91.7 \pm 1.1 \%$  in the control treatment to  $4.0 \pm 1.4 \%$  in the Pitcher GR<sup>®</sup> treatment (Fig. 3b).

#### 4.1.3 Combined Contact and Fumigant Toxicity Bioassay

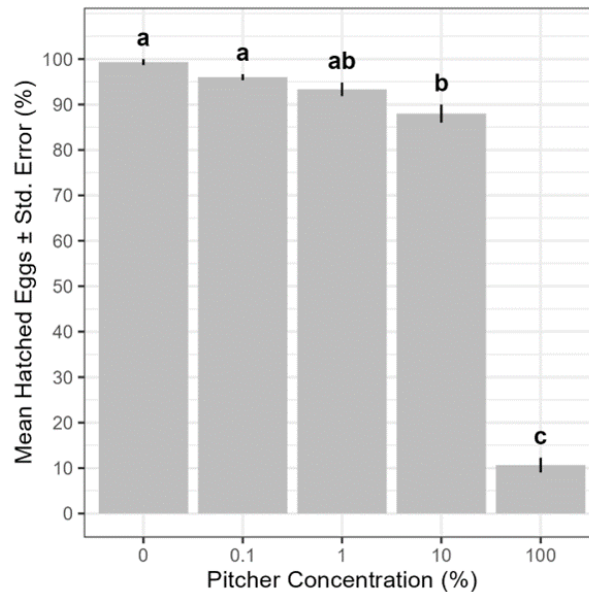
Exposing vine weevil eggs to Pitcher GR<sup>®</sup> in a contact fumigation toxicity bioassay significantly reduced egg hatch rate compared to those exposed to water (GLM with a quasi-Poisson distribution:  $t = -5.678$ ,  $df = 18$ ,  $p < 0.001$ ). The mean percentage of hatched eggs decreased from  $97.3 \pm 1.2 \%$  in the control treatment to  $1.7 \pm 1.7 \%$  in the Pitcher GR<sup>®</sup> treatment (Fig. 3c).

#### 4.1.4 Dose Response Toxicity Bioassay

Exposing vine weevil eggs to varying concentrations of Pitcher GR<sup>®</sup> in a contact fumigation toxicity bioassay significantly reduced egg hatch rate compared to those exposed to water (GLM with a quasi-Poisson distribution:  $t = -49.61$ ,  $df = 23$ ,  $p < 0.001$ ). When applied at the recommended concentration (100 %) Pitcher GR<sup>®</sup> reduced egg hatch to  $10.7 \pm 1.6 \%$ , whereas egg hatch rate was  $88.0 \pm 2.0 \%$ ,  $93.3 \pm 1.5 \%$ ,  $96.0 \pm 0.6 \%$  and  $99.3 \pm 0.6 \%$  at 10 %, 0.1 %, 0.1 % and 0 % respectively (Fig. 4).



**Figure 3.** Percentage (mean ± Std. Err.) of hatched eggs recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) in contact (a), fumigant (b) and combined contact and fumigant (c) tests. Thirty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR<sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR<sup>®</sup>) and untreated group (Control) (GLMs with a quasi-Poisson or Poisson distribution: \*\*\* =  $p < 0.001$ ; NS = not significant).



**Figure 4.** Percentage (mean  $\pm$  Std. Err.) of hatched eggs recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) at different concentrations (0 %, 0.1 %, 1 %, 10 % and 100 %) in combined contact and fumigant test. Thirty eggs (number of replicates = 5) were used for testing the effect of Pitcher GR<sup>®</sup> on vine weevil. Different letters indicate significant differences between tested Pitcher GR<sup>®</sup> concentrations (GLM with a quasi-Poisson distribution:  $p < 0.05$ ).

## 4.2 Vine Weevil Larvae

### 4.2.1 Contact Toxicity Bioassay

Exposing vine weevil larvae to Pitcher GR<sup>®</sup> in a direct contact toxicity bioassay significantly increased larval mortality compared to those exposed to water (GLM with a binomial distribution:  $z = -3.239$ ,  $df = 58$ ,  $p = 0.0012$ ). The mean percentage of live larvae for the control treatment was  $96.7 \pm 3.3$  % while the Pitcher GR<sup>®</sup> treatment mean percentage of live larvae was  $46.7 \pm 8.9$  % (Fig. 5a).

### 4.2.2 Fumigant Toxicity Bioassay

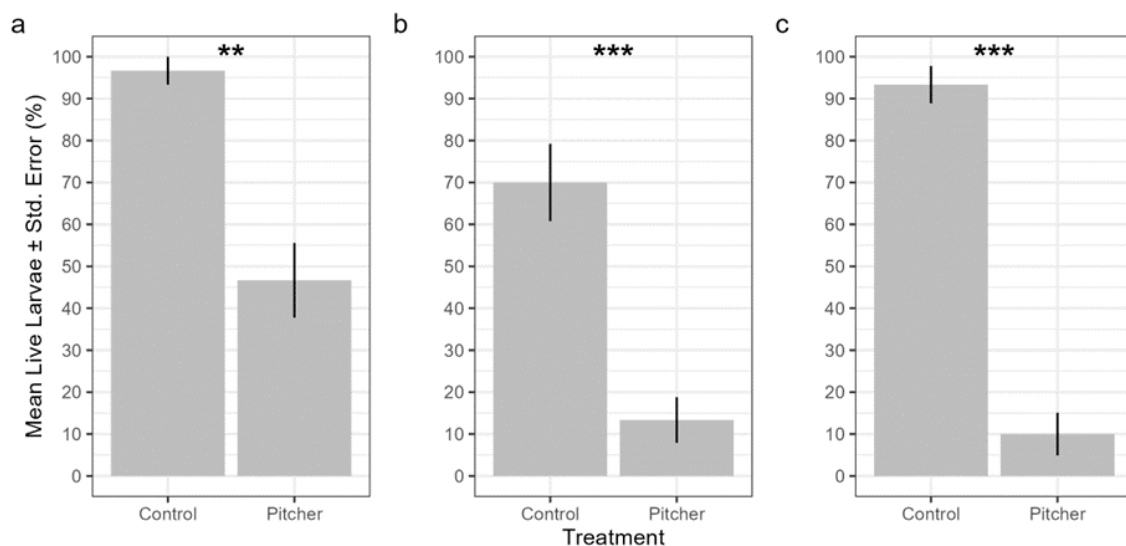
Exposing vine weevil larvae to Pitcher GR<sup>®</sup> in a fumigation toxicity bioassay significantly increased larval mortality compared to those exposed to water (GLM with a binomial distribution:  $z = -4.066$ ,  $df = 58$ ,  $p < 0.001$ ). The mean percentage of live larvae for the control treatment was  $70.0 \pm 9.2$  % while the Pitcher GR<sup>®</sup> treatment mean percentage of live larvae was  $13.3 \pm 5.4$  % (Fig. 5b).

### 4.2.3 Combined Contact and Fumigant Toxicity Bioassay

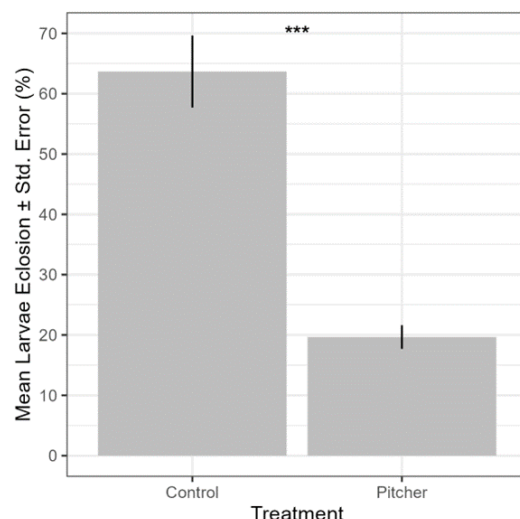
Exposing vine weevil larvae to Pitcher GR<sup>®</sup> in a contact fumigation toxicity bioassay significantly increased larval mortality compared to those exposed to water (GLM with a binomial distribution:  $z = -5.081$ ,  $df = 58$ ,  $p < 0.001$ ). The mean percentage of live larvae for the control treatment was  $93.3 \pm 4.4$  % while the Pitcher GR<sup>®</sup> treatment mean percentage of live larvae was  $10.0 \pm 5.1$  % (Fig. 5c).

### 4.3 Combined Contact and Fumigant Bioassay with Growing Media

Exposing vine weevil eggs to Pitcher GR<sup>®</sup> in a contact fumigation toxicity bioassay under semi-field conditions significantly reduced larval eclosion rate compared to those exposed to water (GLM with a quasi-Poisson distribution:  $t = -7.415$ ,  $df = 18$ ,  $p < 0.001$ ). The mean percentage of larval eclosion decreased from  $62.7 \pm 5.9$  % in the control treatment to  $19.7 \pm 1.9$  % in the Pitcher GR<sup>®</sup> treatment (Fig. 6).



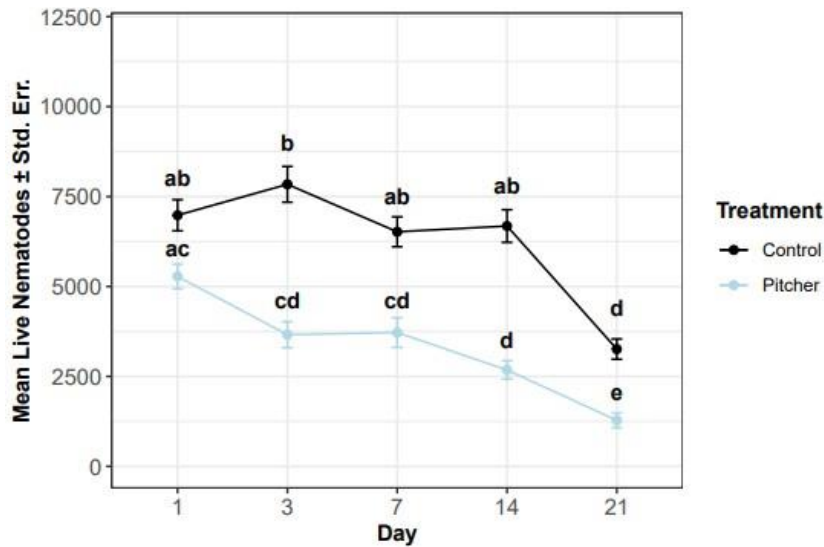
**Figure 5.** Percentage (mean  $\pm$  Std. Err.) of live larvae recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) in contact (a), fumigant (b) and combined contact and fumigant (c) tests. A single larva (number of replicates = 30) was used for testing the effect of Pitcher GR<sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR<sup>®</sup>) and untreated group (Control) (GLM with a binomial distribution: \*\* =  $p < 0.05$ ; \*\*\* =  $p < 0.001$ ).



**Figure 6.** Percentage (mean  $\pm$  Std. Err.) of larvae eclosion recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) in combined contact and fumigant test with growing media. Thirty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR<sup>®</sup> on vine weevil. Asterisks indicate significant differences between treated (Pitcher GR<sup>®</sup>) and untreated group (Control) (GLM with a quasi-Poisson distribution: \*\*\* =  $p < 0.001$ ).

#### 4.4 Compatibility Bioassay

Exposing the entomopathogenic nematode, *S. kraussei*, to Pitcher GR<sup>®</sup> in a contact fumigation toxicity bioassay significantly reduced the number of nematodes compared to the control group (GLM with a quasi-Poisson distribution:  $t = -2.79$ ,  $df = 140$ ,  $p = 0.0059$ ). On Day 1 the mean number of nematodes was  $6980 \pm 21.57$  in the control treatment, while in the Pitcher GR<sup>®</sup> treatment was  $5280 \pm 18.76$  (Fig. 7). On Day 3 the mean number of nematodes was  $7840 \pm 22.86$  in the control treatment, while in the Pitcher GR<sup>®</sup> treatment was  $3660 \pm 15.62$  (Fig. 7). On Day 7 the mean number of nematodes was  $6520 \pm 20.85$  in the control treatment, while in the Pitcher GR<sup>®</sup> treatment was  $3720 \pm 15.75$  (Fig. 7). On the 14 the mean number of nematodes was  $6680 \pm 21.10$  in the control treatment, while in the Pitcher GR<sup>®</sup> treatment was  $2680 \pm 13.37$  (Fig. 7). On Day 21 the mean number of nematodes was  $3260 \pm 14.74$  in the control treatment, while in the Pitcher GR<sup>®</sup> treatment  $1280 \pm 9.24$  (Fig. 7).



**Figure 7.** Mean ( $\pm$  Std Err.) of live nematodes recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) in combined contact and fumigant test over the time (Day: 1, 3, 7, 14 and 21). Different letters indicate significant differences between treatments (Pitcher GR<sup>®</sup>, light blue line; Control, black line) and days (GLM with a quasi-Poisson distribution: Tukey's HSD test:  $p < 0.05$ ).

## 4.5 Glasshouse Experiments

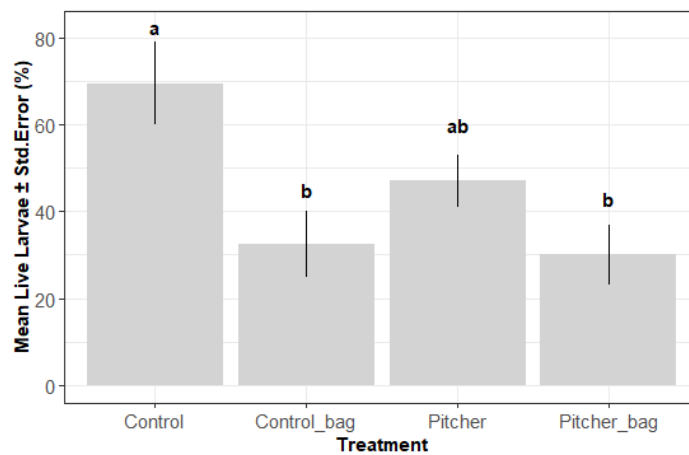
### 4.5.1 Combined Contact and Fumigant Bioassay

Exposing vine weevil eggs to Pitcher GR<sup>®</sup> in a combined contact and fumigation toxicity bioassay under glasshouse conditions did not affect the percentage of larvae (GLM with a quasi-Poisson distribution:  $F^2 = 665.22$ ,  $df = 36$ ,  $p > 0.05$ ) recovered from *Primula* potted plants. When plants were covered with plastic bags the mean percentage of larvae was  $32.5 \pm 6.64$  % in the control treatment compared to  $30 \pm 6.38$  % in the Pitcher GR<sup>®</sup> treatment, whereas without covering the plants the percentage of larvae was  $69.5 \pm 9.7$  % in the control treatment compared to  $47 \pm 7.98$  in the Pitcher GR<sup>®</sup> treatment (Fig. 8).

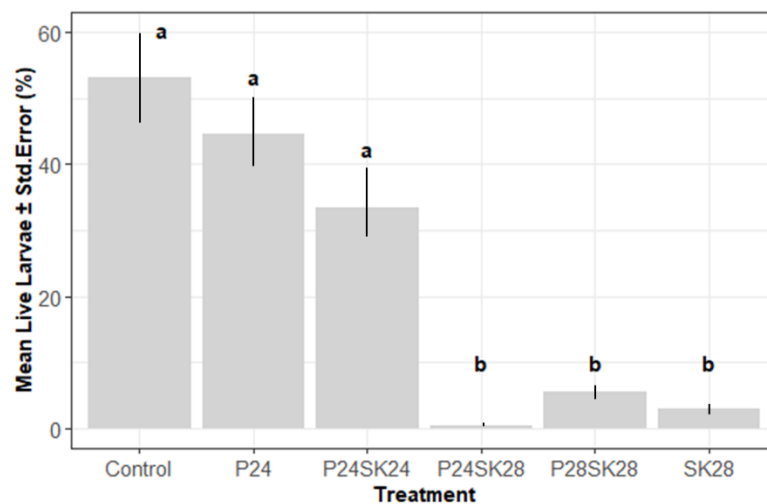
### 4.5.2 Efficacy Bioassay

Exposing vine weevil eggs to different combinations of Pitcher GR<sup>®</sup> and *S. kraussei* in an efficacy bioassay under glasshouse conditions affected the percentage of larvae (GLM with a Poisson distribution:  $F^2 = 606.99$ ,  $df = 54$ ,  $p < 0.001$ ) recovered from *Begonia* potted plants. The number of larvae decreased significantly from  $53 \pm 6.73$  % in the control treatment to  $5.5 \pm 2.17$  %,  $3 \pm 1.60$  % and  $0.5 \pm 0.65$  % in the Treatments 4 (Pitcher GR<sup>®</sup> applied 24 hrs and *S. kraussei* 28 days after eggs= P24SK28), 5 (Pitcher GR<sup>®</sup> and *S. kraussei* applied 28

days after eggs = P28SK28) and 6 (*S. kraussei* applied 28 days after eggs = SK28) (Tukey's HSD test:  $p < 0.05$ ; Fig. 9), respectively.



**Figure 8.** Percentage (Mean  $\pm$  Std. Err.) of live vine weevil larvae recorded when testing the effect of a garlic-based product (Pitcher GR<sup>®</sup>) in combined contact and fumigant test under glasshouse conditions. Different letters indicate significant differences between different treatments (GLM with a quasi-Poisson distribution; Tukey's HSD test:  $p < 0.05$ ).



**Figure 9.** Percentage (mean  $\pm$  Std. Err.) of live larvae recorded when testing the effect of different combinations between a garlic-based product (Pitcher GR<sup>®</sup>) and an entomopathogenic nematode, *S. kraussei*, in an Efficacy Bioassay. For each experimental unit (Begonia plant) twenty eggs (number of replicates = 10) were used for testing the effect of Pitcher GR<sup>®</sup> and/or *S. kraussei* on vine weevil. Different letters indicate significant differences between the treatments (GLM with a quasi-Poisson distribution; Tukey's HSD test:  $p < 0.05$ ). P24 = Begonia with eggs and Pitcher (applied 24hrs after the eggs were applied) no EPNs; P24SK24 = Begonia with eggs and Pitcher and EPNs (applied 24hrs after the eggs were applied); P24SK28 = Begonia with eggs and Pitcher and EPNs (pitcher applied 24hrs after eggs were applied and EPNs applied 28 days after eggs applied); P28SK28 = Begonia with eggs and Pitcher and EPNs (applied 28 days after the eggs were applied); SK28 = Begonia with eggs and EPNs (applied 28 days after eggs were applied) no Pitcher.

## 5. Discussion

Concerns regarding the hazard posed by the use of synthetic chemical insecticides to human and environmental health have led to the development of alternative pest management approaches in agricultural production systems (Souto *et al.*, 2021). Natural products such as phytochemicals derived from plants offer a potential solution to address this need and it is widely believed that they can play an important role in sustainable agriculture (Campos *et al.*, 2019; Lengai *et al.*, 2020). This study demonstrated that the garlic-based Pitcher GR<sup>®</sup> bioinsecticide is effective against vine weevil eggs and larvae under laboratory conditions. In particular, this garlic-based product reduced egg hatch to 4 % compared to the control group (91.7 %) when applied as fumigant. Similarly, egg hatch rate was reduced to 1.7 % compared to the control group (97.3 %) when garlic was applied as combined contact and fumigant applications. No effect was observed when the garlic product was applied as a contact only application. The bioinsecticide significantly reduced larval survival to 46.7 % and 13.3 % when either contact or fumigant applications were used, respectively.

These results are in line with previous studies where naturally derived products have been shown to be effective against several economically important weevil (Curculionidae) pests. For example, neem seed extract containing 4.5 % azadirachtin has been reported to reduce the survival of the root weevil (*Diaprepes abbreviatus* L.) larvae in laboratory and greenhouse experiments (Weathersbee and Tang, 2002). Similarly, Parwada *et al.* (2018) reported the lethal effect of three botanical pesticides, the forest red gum (*Eucalyptus terreticonis* Sm; Myrtales: Myrtaceae), the mint marigold (*Tagetes minuta* L.; Asterales: Asteraceae) and the common lantana (*Lantana camara* L.; Lamiales: Verbenaceae), against the maize weevil (*Sitophilus zeamais* Motschulsky). Powders, extracts, essential oils and purified compounds from garlic have been reported to have lethal and sub-lethal effects to a number of pest species from the Coleoptera, Lepidoptera, Hemiptera and Diptera insect orders (Huang *et al.*, 2000; Yang *et al.*, 2012; Ramzi *et al.*, 2022). Acetone leaf extracts of garlic have been shown, for example, to reduce survival of all red flour beetle (*Tribolium castaneum* Herbst; Coleoptera: Tenebrionidae) life-cycle stages under laboratory conditions (Ali *et al.*, 2014). Similarly, volatile chemicals associated with garlic were recorded to be effective against adults of the cowpea weevil (*Callosobruchus maculatus* Fabricius; Bruchidae: Coleoptera) at all the concentrations tested between 0.1 % and 20 % (Abdalla *et al.*, 2017). Under glasshouse conditions, a garlic extract product (Ecoguard-granules; Ecospray Limited) at a dose equivalent to the rate of 0.12 g product per plant has been shown to reduce by almost 60 % the numbers of cabbage root fly (*Delia radicum* L.; Diptera: Anthomyiidae) larvae/pupae (Jukes *et al.*, 2005).

The reduction in egg hatch due to the fumigant and contact activity of garlic-based products has been previously documented (Ekesi, 2000). For example, Ho *et al.* (1996) showed that oil from fresh garlic cloves caused mortality in red flour beetle eggs and that eggs were the most susceptible stage compared to adults, ten-day-old larvae and older larvae. In another study, two of the major constituents of garlic oil, namely methyl allyl disulfide and diallyl trisulfide, have been also reported to reduce egg hatching of the red flour beetle (Huang *et al.*, 2000). Prowse *et al.* (2006) reported that a commercial garlic juice-based insecticide was effective against the cabbage root fly (*Delia radicum* L.; Diptera: Anthomyiidae) and the housefly (*Musca domestica* L.; Diptera: Muscidae) eggs. However, it was not fully understood if the reduction in egg hatch was due to contact or fumigant toxicity. Garlic extracts are believed to contribute to increase insect mortality by preventing larval eclosion (Jarial, 2001). Some bioactive components from garlic are thought to cause lethal physiological changes during embryonic development, so that hatching is inhibited (Prowse *et al.*, 2006). These findings are promising and suggest the potential of garlic-based products as possible strategy for pest control.

Vine weevil larval survival was reduced, in the present study, when the garlic-based product was applied in a way that contact, fumigant or both contact and fumigant effects were possible. This study did not investigate the underlying modes of action, however, previous studies have reported that some enzymes such as acetylcholinesterase, which regulates acetylcholine levels in the nervous system, can be inhibited by garlic extracts (Bhatnagar-Thomas and Pal, 1974; Singh and Singh, 1996). In addition, garlic constituents have been shown to affect the respiration rate, leading to physiological stress and paralysis of insects (Plata-Rueda *et al.*, 2017). Allicin, the major component present in garlic, has been reported to cause embryonic death as well as affect fertility, fecundity and the production process of moulting hormones (Mamduh *et al.*, 2017). Despite this, the modes of action of garlic extracts are still not fully understood and further studies are, therefore, needed.

The efficacy of garlic and its constituents in controlling insect pests has been shown to be affected by a variety of biological factors, including pest development stage. For example, Plata-Rueda *et al.* (2017) showed that the mealworm beetle (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae) was more susceptible to two garlic compounds, diallyl sulfide and diallyl disulfide, in the pupal stage, with the larval and adult stages less susceptible. The authors suggested that efficacy may be influenced by the penetration of the garlic compounds into the body of the insect and the ability of the insect to metabolize these compounds. However, further investigation into how garlic affects insect pests at different developmental stages is needed.

Although garlic-based oils and extract formulations have been studied in many laboratory tests, relatively few studies present results from its practical use. This study aimed

to investigate the efficacy of Pitcher GR<sup>®</sup> also under glasshouse conditions. Results showed that this bioinsecticide was not effective in reducing the number of larvae recovered from potted Begonia or Primula plants compared to the control group. Several studies have shown that the efficacy of plant extracts in the field is variable and extremely determined by prevailing environmental conditions (Lengai *et al.*, 2020). These include temperature, relative humidity, level of sunlight radiation, altitude, photoperiod and type of soil as well as ecological interactions (e.g., herbivory or mutualism) (Souto *et al.*, 2021). Consequently, variables influencing the efficacy of plant extracts, such as Pitcher GR<sup>®</sup>, need to be further investigated under commercial field conditions, to better understand the potential of their use as alternatives to synthetic chemical pesticides. In addition, it is believed that egg and larval stages are most susceptible to botanical bioinsecticides (Erlor *et al.*, 2010; Kedia *et al.*, 2015). If true, this would mean that like other biopesticides, application timing would be important to effectively target these stages of vine weevil life cycle (Pope and Roberts, 2022). The method of application is also likely to be crucial in targeting these stages as this will determine whether contact or fumigant activity is achieved. Additional research is also necessary to investigate the effect of garlic on other vine weevil life stages, such as late instar larvae, pupae and adults.

There is currently a lack of knowledge on the efficacy of botanical bioinsecticides against below-ground insect pests such as weevils. This is despite the fact that some botanical bioinsecticides, such as neem (*Azadirachta indica* A. Juss; Sapindales: Meliaceae), have been used as soil amendments against plant parasitic nematodes (Abbasi *et al.*, 2005) and fungal pathogens (Khan *et al.*, 2020). Further studies are, therefore, required to improve the use of such products, including Pitcher GR<sup>®</sup>, against weevil pests and promote their incorporation into IPM programmes.

In this study the compatibility between the commercially available garlic-based product and the EPN, *S. kraussei*, was investigated under laboratory condition. Results indicate that when the recommended rate of the bioinsecticide was applied simultaneously with *S. kraussei*, the garlic-based product reduced the survival of the EPN compared to the control. Therefore, these results indicate a poor compatibility between the tested product and the EPN under the tested conditions. A similar result was reported by Meyer *et al.* (2012) when investigating the effects of commercial neem formulations on EPNs. The direct exposure of *H. bacteriophora* to NeemAza<sup>®</sup>-U (a formulation containing 17% azadirachtin) for fourteen days resulted in 14 % mortality compared to 6.5 % in the control. Similarly, lethal effects of Margosan-O, a commercial product of neem seed extract, have been reported on Steinernematids (Stark, 1996). In particular, *S. feltiae* (Filipjev) has been shown to be more susceptible than *S. glaseri* (Steiner) or *S. carpocapsae* (Weiser) (Stark, 1996). Other studies have reported the toxicity of clove (*Syzygium aromaticum*, Family: Myrtaceae), cinnamon (*Cinnamomum verum*,

Family: Lauraceae) and garlic oils to EPNs (Meyer *et al.*, 2008; Gupta *et al.*, 2011; Barua *et al.*, 2020).

The compatibility of botanical bioinsecticide formulations with EPNs seems to differ with species, strains, doses, adjuvants used in formulations as well as formulations and application dose of pesticides (Krishnayya and Grewal, 2002; Gutiérrez *et al.*, 2008; Laznik and Trdan, 2014; Reddy and Chowdary, 2021). Therefore, it is important for each bioinsecticide-EPN species combination to be tested before recommendations for use as part of IPM programme are given. This compatibility may promote the combined use of EPNs and bioinsecticides as a cost-effective pest control strategy.

After 21 days exposure to Pitcher GR<sup>®</sup>, surviving *S. kraussei* (1280 ± 9) were still found under the tested laboratory conditions. No studies were carried out to test if the nematodes exposed to the garlic-based product would be still able to infect vine weevil larvae. Campos-Herrera *et al.* (2023) noted that the compatibility between EPNs and pesticides requires more than just the survival of EPNs. De Nardo and Grewal (2003) showed that although the survival of *S. feltiae* exposed to the pesticide Gnatrol (*Bacillus thuringiensis* subsp. *israelensis*) decreased to 17 % after seventy-two hours of incubation, however, the surviving infective juveniles showed higher infectivity to the greater wax moth (*Galleria mellonella*, Linnaeus; Family: Pyralidae) larvae. Similarly, Rovesti and Deseö (1990) assessing the compatibility of *S. carpocapsae* and *S. feltiae* to commercial pesticides, reported that pesticides such as organophosphorus compounds and endosulfan reduced the movement of these infective juveniles, but infectivity was not affected. Finally, Oso *et al.* (2021) showed that the survival of *H. bacteriophora* was low (below 20 %) when exposed to an aqueous extract of the Larger Tinsel Flower (*Alepidea amatymbica*, Eckl. and Zeyh.; Family: Apiaceae), but again, the nematode was still able to infect yellow mealworm beetle (*Tenebrio molitor*, Linnaeus; Tenebrionidae) larvae. The diversity of susceptibility and sensitivity to different pesticide formulations to EPNs might be attributed to the differentiation of nematodes, insect physiology, feeding patterns and pesticide doses. In this context, the role of nematode physiology in reducing susceptibility to various pesticides, including botanical bioinsecticides, requires further investigation.

Although some bioinsecticides may affect EPNs persistence and infectivity (Zimmerman and Cranshaw, 1990), a study conducted by Rovesti and Deseö (1990) showed that nematodes were tolerant to short periods (e.g., from 2 to 4 hours) of exposure to the majority of seventy-five commercial pesticides tested. This means that incompatibility may be managed by choosing an appropriate time-intervals between nematode and pesticide applications. In this context, the results in this study showed that combining Pitcher GR<sup>®</sup> and *S. kraussei* at different times (e.g. applied twenty-four hours and twenty-eight days after vine weevil application, respectively) was effective in controlling vine weevil larvae. However, the

use of the EPN alone (e.g., twenty-eight days after vine weevil application) was effective in reducing the number of larvae recovered from Begonia plants compared to the control group. Further studies are needed to understand and determine compatibility, the types of interactions between these two strategies as well as the time intervals between applications before integrating in a IPM programme for vine weevil control.

This study demonstrates the potential of garlic-based bioinsecticides such as Pitcher GR<sup>®</sup> to suppress vine weevil egg and larval development in controlled conditions. The product's multiple toxic effects (contact, fumigant and both combined toxicity), however, were seen under laboratory but not glasshouse conditions. In addition, Pitcher GR<sup>®</sup> does not appear to be compatibility with the EPN, *S. kraussei* when applied simultaneously. Therefore, future studies are needed to determine whether the potential of garlic-based bioinsecticides can be realised as a sustainable vine weevil management tool.

# Chapter 6: Efficacy of Entomopathogenic Nematodes in Peat-Free Growing Media

## Abstract

Entomopathogenic nematodes (EPNs) are an important tool for controlling insect pests in agriculture and horticulture. Despite this, their efficacy has been shown to be affected by many factors (e.g., temperature, humidity and growing media) and there has been limited focus on the effect of peat-free growing media on EPNs performance, which is becoming a critical concern as the horticultural industry shifts away from peat-based growing media. In this study, the survival, dispersal and parasitism rate of *Steinernema kraussei* (Steiner; Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* (Polinar; Rhabditida: Heterorhabditidae) infective juveniles (IJs) were investigated in six commercially available peat alternative growing media under laboratory conditions. Survival and dispersal of IJs was assessed directly by counting living IJs. Parasitism rate was measured by change of colour and the presence of nematodes within vine weevil larvae. In addition, under glasshouse conditions, the efficacy of *S. kraussei* and *H. bacteriophora* against vine weevil larvae was assessed using Begonia (*Begonia x semperflorens-cultorum*, Hort; Family: Begoniaceae) plants in six peat-free growing media. Although, nematode survival significantly decreased over time under laboratory conditions, EPNs were able to survive in all growing media after twenty-eight days post application. Survival of *S. kraussei* was higher than for *H. bacteriophora*. Dispersal of EPNs was influenced by growing media, with Coir and John Innes Number 2 having a negative impact upon *H. bacteriophora* and *S. kraussei* dispersal. Parasitism rate was not significantly impacted by growing media for either EPN species. Under glasshouse conditions, only *S. kraussei* was effective at controlling vine weevil larvae in all the tested growing media compared to the control group, except in Coir and Multipurpose growing media. In addition, larvae recovered from Begonia grown in Coir growing media weighted less compared to those recovered from other growing media types.

This study is the first to highlight the importance of peat-free growing media type on EPN efficacy. Despite some differences being apparent, results indicate that *S. kraussei* provides a good option for vine weevil management regardless of the peat-free growing media used.

## 1. Introduction

Entomopathogenic nematodes (EPNs) are insect-parasitic roundworms that kill their hosts by septicemia and toxemia (Karthik Raja *et al.*, 2021). Third-stage juveniles (infective juveniles (IJ; IJ<sub>3</sub>)) are free-living and reside in soil with non-foraging behaviour. Once the infective juveniles (IJs) penetrate the host body, through spiracles or through natural body openings, they release their symbiotic bacteria (e.g., *Xenorhabdus* and *Photorhabdus* for *Steinernema* and *Heterorhabditis*, respectively) inside the haemocoel of the insect host that results in host death (Lewis and Clarke, 2012). The bacterial cells replicate and produce toxins that have insecticidal properties that can result in death of the host in less than two days (Adams and Nguyen, 2002). Within the insect cadaver, these bacteria nourish the EPNs and promote the growth and reproduction of EPNs. Eventually, a new generation of IJs emerges from the cadaver and begins searching for new hosts.

Nematodes belonging to *Heterorhabditis* (Rhabditida: Heterorhabditidae) and *Steinernema* (Rhabditida: Steinernematidae) genera with at least 117 described species (17 *Heterorhabditis* and 100 *Steinernema* species) (Bhat, Chaubey and Askary, 2020) have been shown to be effective in managing key insect pest populations (Kaya and Gaugler, 1993; Singh *et al.*, 2022). In the field of integrated pest management (IPM), EPNs have been shown to control economically important pests such as the codling moth (*Cydia pomonella*, L.; Lepidoptera: Tortricidae) (Lacey *et al.*, 2006), cabbage root fly (*Delia radicum*, L.; Diptera: Anthomyiidae) (Kapranas *et al.*, 2017), the root weevil (*Diaprepes abbreviatus*, L.; Coleoptera: Curculionidae) (Bender *et al.*, 2014), the vine weevil (*Otiorhynchus sulcatus*, Fabricius; Coleoptera: Curculionidae) (Ansari *et al.*, 2008), the garden chafer (*Phyllopertha horticola*, Linnaeus; Coleoptera: Scarabaeidae) (Smits, Wieggers and Vlug, 1994), *Thrips* spp. (Ebssa *et al.*, 2004). However, the efficacy of EPNs is affected by various abiotic (i.e., temperature, moisture, growing media texture, growing media salinity, UV light, oxygen and pH) and biotic (i.e., natural enemies, omnivores, scavengers, competitors and plants) factors (Stuart *et al.*, 2015). For example, Chen *et al.* (2003) investigating the susceptibility of the cabbage root fly (*Delia radicum* L.; Diptera: Anthomyiidae) last instar larvae to *S. feltiae*, *S. carpocapsae*, *S. arenarium*, *Heterorhabditis megidis* and *H. bacteriophora* showed that temperature significantly affected the host searching ability of all the tested species.

Several studies have also reported the influence of the growing media on EPNs efficacy (Kaya, 1990). The physico-chemical properties that more affect EPNs efficacy include growing media texture (Khumalo *et al.*, 2021), porosity, bulk density (Kapranas *et al.*, 2017) and moisture content (Salame and Glazer, 2015). For example, soil or growing media texture and pore size can influence EPNs movement, their host finding ability and survival (Kaya and Gaugler, 1993). In particular, it has been shown that the dispersal of EPNs, whether horizontal

or vertical, as well as their pathogenicity and survival decrease with the increase in the overall proportion of silt and clay (Kung *et al.*, 1990).

While many studies have investigated the efficacy of EPNs in different growing media, these have mainly been growing media containing peat. Peat itself is partially decomposed organic matter sourced from wetlands and peat bogs (Kern *et al.*, 2017). In recent decades peatlands have been recognised as an important store of carbon and extraction of peat releases greenhouse gases, thereby contributing to climate change (Ribeiro *et al.*, 2021). As consequence, government policies are shifting towards reducing the use of peat-based growing media. For example, the United Kingdom has set a goal to ban peat in the amateur horticulture sector by 2024 (Defra, 2021). Similarly, the Royal Horticultural Society aims to become peat-free by 2025 (The Royal Horticultural Society, 2021). Hence, it is important for research to focus on finding alternatives to peat-based growing media and investigate, for example, whether the efficacy of EPNs in controlling pest insects remains effective and reliable.

The aim of this study is to assess the survival, dispersal and parasitism rate of two EPN species, *S. kraussei* and *H. bacteriophora*, in six commercially available peat-free growing media under laboratory conditions. Similarly, the efficacy of EPNs in controlling vine weevil was assessed in peat-free growing media under glasshouse conditions. It is hypothesised that: (i) growing media type influences the dispersal, parasitism rate and survival of EPNs and (ii) there is a difference in the performance of *H. bacteriophora* and *S. kraussei*.

## **2. Materials and Methods**

### **2.1 Insect Cultures**

Vine weevil adults and larvae were collected from commercial strawberry (*Fragaria × ananassa* cv. *Duchesne*) crops grown in Staffordshire (UK). Ten larvae were placed into individual plastic containers (17 x 10.2 x 3 cm, GP Globe Packaging, UK) containing soil (SylvaGrow® John Innes Number 2, Tetbury, UK) and carrot slices as a food source. These were maintained in a controlled environment room (20 °C; 60 % relative humidity) (Fitotron, Weiss Technik, Ebbw Vale, Wales, UK) under complete darkness. Thirty adults were placed into plastic terrarium (30 × 19.3 × 20.6 cm, Exo Terra, Castleford, UK) containing winter creeper (*Euonymus fortunei* Emerald n Gold Turcz. Hand.-Maz.; Celastrales: Celastraceae) branches, cotton wool balls and moist paper towels. These were maintained in a controlled environment room (20 °C; 60 % relative humidity; 16:8 h light:dark photoperiod; Fitotron). Eggs were collected every three days from the cotton wool balls placed in each terrarium using a

fine 000 paintbrush. All eggs were examined under a binocular microscope (Microtec HM3, Somerset, UK) to ensure that only undamaged, fully melanised eggs were used for an experimental batch. These experimental batches (approximately 100 eggs) were then stored at 5 °C (Lec LSR151, Merseyside, UK) in plastic containers (115 x 40 mm, Signature Packaging, UK) until they were used in a bioassay. Eggs were used in bioassays within seven days of collection from the terraria. Regular egg viability counts were carried out by maintaining twenty eggs on moist tissue paper in a 90 mm diameter plastic Petri dish (Thermo Scientific™ 101VR20, Fisher Scientific UK Ltd) in a controlled environment room (Fitotron) at 20 °C and 60 % relative humidity in complete darkness.

## 2.2 EPN Dilution

The nematodes Nemasys® (*S. kraussei*, BASF, UK) and Nema-green® (*H. bacteriophora*, e-nema GmbH, Germany) in packs of 6 million and 50 million, respectively, were used for laboratory experiments. For glasshouse experiments, nematodes were provided by BASF (Stockport, UK), using Nemasys® L (*S. kraussei*) and Nemasys H® (*H. bacteriophora*) in packs of 50 million. Nematodes were stored at 5.0°C (Lec LSR151, Merseyside, UK). A nematode stock solution for laboratory or glasshouse experiments was prepared by diluting a single pack of nematodes in tap water (e.g., 2 Liters for packs containing 50 million nematodes and 1.2 Liters for packs containing 6 million). The solution was aerated for five minutes using a Tetra APS 50 air pump (Malle, Germany). To count viable nematodes, 1 ml of the stock solution was taken and diluted with tap water up to 200 ml. From this dilution, 1 ml was pipetted into a 3 × 5 cm plastic counting chamber with gridlines and live infective juveniles (IJs) were counted under a dissecting microscope (Microtec HM3, Somerset, UK). The count was done in triplicate and the entire process was repeated three times (for a total of nine counts) to estimate the total number of live IJs in the stock solution. Based on this, the desired concentrations were created to complete laboratory and glasshouse bioassays.

## 2.3 Growing Media

The peat alternative growing media used for laboratory and glasshouse experiments were Cocogreen® Berryfusion™ Growbags (= Coir; Manchester, UK) and five SylvaGrow® (Melcourt® Industries Limited, UK) growing media, namely Multipurpose (MP), with added John Innes (JI), John Innes Number 2 (John Innes N. 2), Tub and Basket (TB) and Organic Planter (OP). Properties of the growing media were either provided by the supplier or

determined experimentally (Table 1). To set the moisture levels, 200 g of the growing media was submerged in tap water for 30 minutes in 1 L glass jars (Teku VCH 13, Pöppelmann, Lohne, Germany). Afterward, the media was removed and drained for one hour in organza bags (13 × 18 cm; Amison, UK) until no more water dripped, achieving full saturation (Hartley and Wallace, 2017). Moisture levels were recorded in laboratory and glasshouse conditions using a WET-2 sensor (Delta-T Cambridge, UK) and SensorPush HT.w Water-Resistant Smart Sensors (New York, USA), respectively (Table 1).

**Table 1.** Chemical and physical properties of the growing media used for laboratory and glasshouse experiments.

Growing media	Components <sup>a</sup>	pH <sup>a</sup>	Bulk Density (g/l) <sup>a</sup>	Moisture (%) <sup>b</sup>		Humidity (%) <sup>c</sup>	Temperature (°C) <sup>c</sup>	
				Dispersal Bioassay	Survival Bioassay		Efficacy Bioassay	Efficacy Bioassay
							Min	Max
<b>SylvaGrow Multipurpose</b>	Fine Bark, Coir, Woodfibre	6.8	160	63	59	99.9	6.2	32.1
<b>SylvaGrow Added John Innes</b>	Fine Bark, Coir, Woodfibre, Sterilised Loam, Sand	7.3	247	63.3	67.7	99.9	6.8	30.8
<b>SylvaGrow John Innes N.2</b>	Fine Bark, Woodfibre, Sterilised Loam	7.7	544	61.4	63	100	6.4	34.1
<b>SylvaGrow Tub and Basket</b>	Fine Bark, Coir, Woodfibre, Water Retaining Aid	7	170	65.5	60.7	97.1	5.5	35.1
<b>SylvaGrow Organic Planter</b>	Fine Bark, Green Compost, Coir	6.6	165	56.7	52.1	98.8	5.9	34.7
<b>Cocogreen Berryfusion</b>	COIR	5.4-6.8	168	46.3	45.7	93	6.6	39.7

<sup>a</sup> Data were provided by the supplier (Melcourt Industries Ltd, UK).

<sup>b</sup> Mean moisture was recorded for the experiments carried out under laboratory conditions.

<sup>c</sup> Temperature and Humidity were recorded for the experiments carried out under glasshouse conditions.

## 2.4 Laboratory Experiments

### 2.4.1 Survival Bioassay

The effect of growing media on the EPN survival was assessed under laboratory conditions. Experimental units (Fig. 1a) consisted of 50 ml plastic pots (Fabri-Kal GPC200, Illinois, USA), each filled with 20 ml of growing media, sealed with airtight lids. At the beginning of the experiment, each pot was weighed. Subsequently, every week, the pots were opened to allow air exchange and water was added to bring each experimental unit back to its original weight (Koppenhöfer and Fuzy, 2006). Pots containing the growing media were inoculated with 500 µl of stock EPN solution (containing 20,000 IJs), with the stock solution prepared as described earlier in Section 2.2 for each growing media type (Table 1) and EPN species. The experimental units were then placed in a controlled environment chamber (Fitotron) at 20 °C in complete darkness. Twenty-four hours after inoculation, the number of live IJs was determined by rinsing the entire contents of one pot with 300 ml of tap water. A 1 ml sample of this solution was then pipetted into a 3 × 5 cm counting chamber with gridlines, and live IJs were counted using a dissecting microscope (Microtec HM3, Somerset, UK). This assessment was conducted in triplicate with the entire procedure repeated three times (resulting in a total of nine counts) to estimate the total number of live infective juveniles (IJs) in each pot. The same process was carried out on days 7, 14, 21, and 28 following nematode application. The entire experiment was replicated five times.

### 2.4.2 Dispersal Bioassay

The effect of growing media on EPN dispersal was assessed under laboratory conditions. Experimental units (Fig. 1b) were similar to those used by Nielsen *et al.* (2011) and consisted of four segments of cross-linked polyethylene (PEX) pipe (2 × 1.5 cm, Screwfix, UK) taped (Electric insulation tape; Duck, UK) together, making 8 cm long tubes (Ansari and Butt, 2011b). Each growing media type (see Section 2.3) was individually added to tubes and lightly pressed with the end of a 000 fine paintbrush (Meeden®, China) to ensure a constant column of growing media (Fig. 1b). In baited experimental units, one vine weevil larva was enclosed with fine mesh in the bottom segment with growing media (Fig. 1b) while control experimental units were not baited with larva. After twenty-four hours, 100 µl of a nematode solution (containing 500 IJs), using the stock solutions for each EPN species previously described in Section 2.2, was added using a micropipette (SciQuip Ltd, UK) to the corresponding experimental units. Each end of the experimental unit was sealed with a double layer of perforated Parafilm to allow for gas exchange. Experimental units were stored

vertically at 20 °C in a controlled environment room (60 % relative humidity; Fitotron) in complete darkness. After twenty-four hours, the final segments were separated from columns. For baited experimental units, each larva was removed from the final segment and washed with 10 ml of tap water, to remove any external IJs, and transferred to 110 ml plastic pots (Fabri-Kal GPC200, Illinois, USA). The contents of the final segments were washed with 70 ml of tap water and added to the rest of the solution in the 110 ml plastic pots. Similarly, for control experimental units, the contents of the final segments were washed with 80 ml of tap water and poured in 110 ml plastic pots (Fabri-Kal GPC200, Illinois, USA). A 1 ml sample of this solution was then pipetted into a 3 × 5 cm counting chamber with gridlines and live IJs were counted using a dissecting microscope (Microtec HM3, Somerset, UK). This was done in triplicate, with the entire procedure repeated three times (resulting in a total of nine counts), so the total number of live IJs in each plastic pot and therefore final segment of the experimental unit could be estimated. Vine weevil larvae were moved into Petri-dishes (60 × 15 mm; Sarstedt AG & Co. KG, Germany) containing 1 ml of tap water and stored at 20 °C (Fitotron) in complete darkness for seven days. Mortality by EPN infection was assessed by observation of colour change. Similarly, a larva was considered infected if at least one IJ was found after dissection. The whole experiment was repeated 20 times.

## **2.5 Glasshouse experiments**

### **2.5.1 Begonia Plants**

Wax Begonia (*Begonia x semperflorens-cultorum*, Hort; Family: Begoniaceae) plants were grown from Begonia seeds (Mr Fothergill's, UK) in seed trays with lids (Nutley's Kitchen Gardens, UK) containing growing media (SylvaGrow® John Innes N. 2, Tetbury, UK). The trays were then maintained for three months in a controlled environment room (20 °C; 60 % relative humidity; 16:8 h light:dark photoperiod) (Fitotron, Weiss Technik, Ebbw Vale, Wales, UK). After this period John Innes N. 2 was gently shaken from the roots of the plants before potting them on in one of the five peat-free growing media (Tab. 1) inside 1 L pots (13 cm diameter; Teku VCH13, Pöppelmann, Lohne, Germany). Plants were left to grow for four weeks under glasshouse conditions (21.04 ± 0.01 °C; 42.9 ± 0.04 % relative humidity) before using in glasshouse experiments. Plants were watered every two to three days as required, by pouring water into plastic saucers (16 cm in diameter; Whitefurze Ltd, UK).

### 2.5.2 Efficacy Bioassay

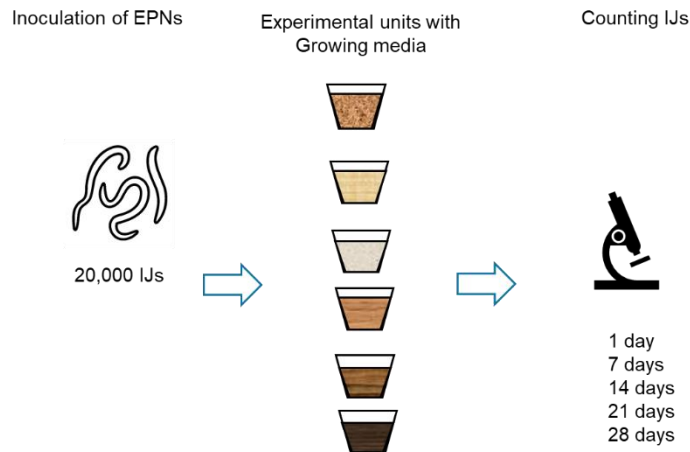
The efficacy of *EPNs* at controlling vine weevil in peat free growing media was assessed under glasshouse conditions. Experimental units (Fig. 1c) consisted of Begonia plants (see Section 2.5.1) potted up in peat alternative growing media. For each growing media, potted plants were treated with one of three treatments: 1) treated with *S. krausseii*; 2) treated with *H. bacteriophora*; 3) not treated with EPNs and 4) not treated with EPNs and not containing vine weevil eggs. Twenty vine weevil eggs (Section 2.1) were applied around the treatment plant stems using a 000 fine paintbrush (Meeden<sup>®</sup>, China). After twenty-eight days, 100 ml of the nematode dilution (25000 IJs), using a stock solution prepared as previously described in section 2.2, was applied to each required EPN-treated potted plant by pouring directly onto the surface of the growing media (which had been watered the day before with 100 ml of water). In the control groups 100 ml of water was applied to each potted Begonia plant. Begonia potted plants were placed in individual plastic saucers (16 cm in diameter; Whitefurze Ltd, UK) to prevent EPN contamination between treatments. The plants were watered every 2-3 days as needed, by adding water into the saucers and ensuring, in this way, that the EPNs were not washed out. Pots were arranged in a randomised block design. Six replicates per each treatment were prepared, including for the control treatment. A viability count of vine weevil eggs was carried out by keeping twenty eggs on moist tissue paper in a 90 mm diameter Petri-dish (Thermo Scientific™ 101VR20, Fisher Scientific UK Ltd) under laboratory conditions (20 °C; 60 % relative humidity; 24 hours of darkness). After twenty-eight days each Begonia plant was checked and the number of live larvae in each pot was recorded. Larvae recovered from each Begonia plant were weighted using an OHAUS Navigator balance (New Jersey, US). Glasshouse temperatures were recorded with SensorPush HT1Smart Sensors (New York, USA) (Table 2). Furthermore, SensorPush HT.w Water-Resistant Smart Sensors (New York, USA) were set up in pots with each growing medium to record temperature and humidity (Table 1).

**Table 2.** Glasshouse temperatures recorded during the Efficacy Bioassay

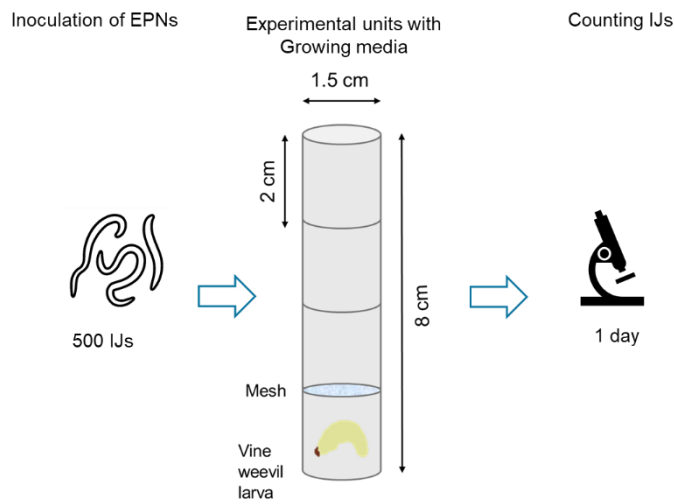
Replicate	Temperature (°C)		
	Min.	Average	Max.
1 & 2	12.8	23.4	28.4
3 & 4	13.3	20.8	27.5
5 & 6	15.5	21.9	31.8

\*Temperatures recorded using SensorPush HT1Smart Sensors (New York, USA).

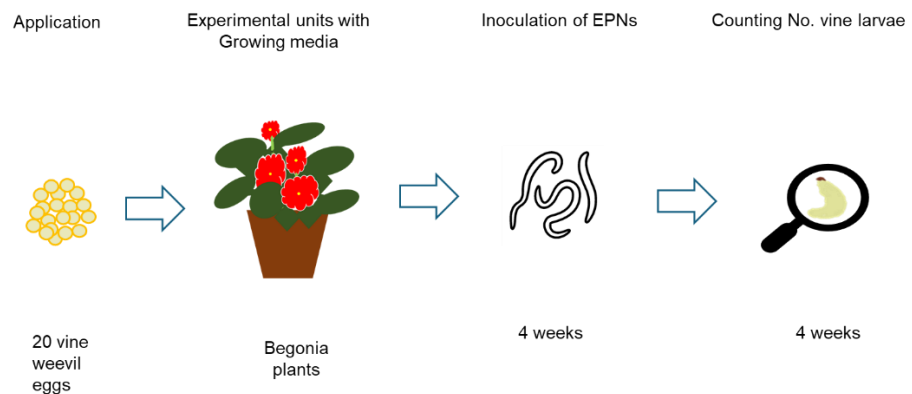
**a) Survival Bioassay**



**b) Dispersal Bioassay**



**c) Efficacy Bioassay**



**Figure 1.** Diagram showing the set-up and the experimental units for the survival experiment (a), the dispersal experiment (b) (under laboratory condition) and the efficacy experiment (c) under glasshouse conditions.

### 3. Statistical Analysis

Statistical analyses were carried out in R v 4.3.2 (R Core Team, 2022). In the Survival and Dispersal Bioassays testing the effect of growing media for both EPNs, count data were analysed using generalised linear models (GLMs) fitted to a Poisson or quasi-Poisson probability distribution depending on the presence of overdispersion. This was determined using `qcc.overdispersion.test` function from the `qcc` package (Scrucca, 2004), where a p-value greater than 0.05 indicated no overdispersion and justifies the use of Poisson distribution. Parasitism rate was analysed using GLM with a binomial distribution, as there were only two outcomes in the bioassays: infected or uninfected. Tukey's Honest Significant Difference (HSD) test ( $p < 0.05$ ) was used to compare the mean values between treatment groups where appropriate. The weight of vine weevil larva exposed to different treatments (*S. kraussei* and controls) in different peat-free growing media was analysed with a non-parametric Kruskal-Wallis test since distribution of the data were deviated from normality (Shapiro-Wilk normality test:  $p > 0.05$ ) followed by pairwise comparisons using the Wilcoxon rank-sum test with Bonferroni's correction.

## 4. Results

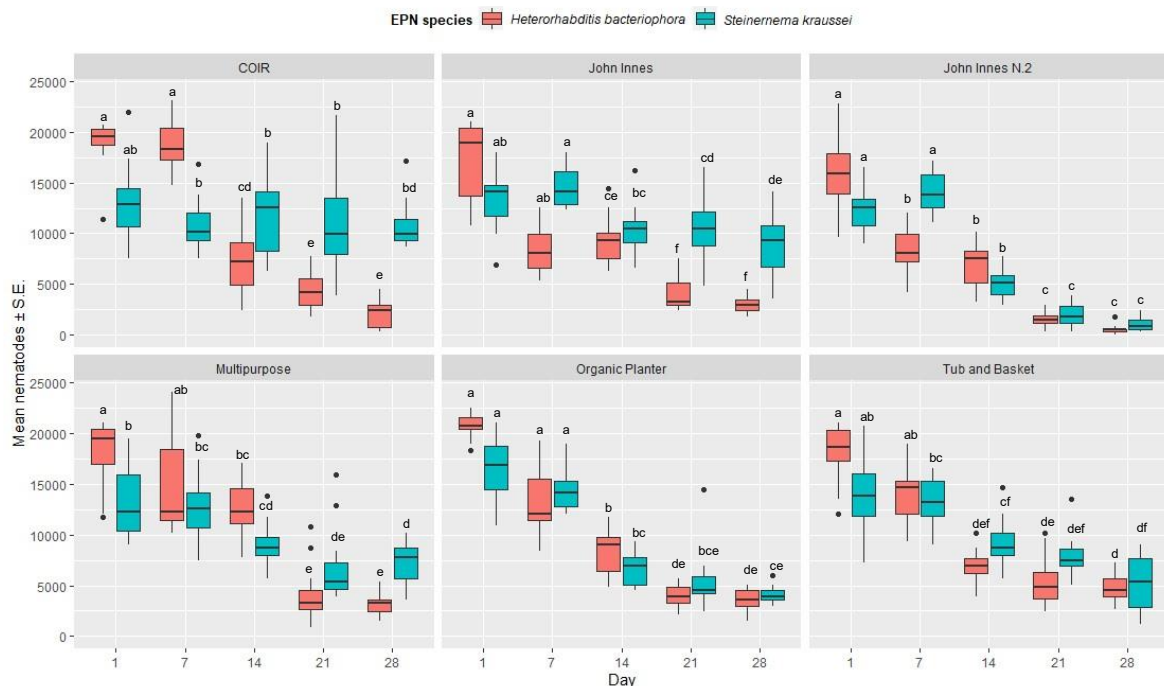
### 4.1 Laboratory Experiments

#### 4.1.1 Survival Bioassay

EPN survival was evaluated in a bioassay consisting of counting the number of *H. bacteriophora* and *S. kraussei* in experimental units with different growing media over a period of twenty-eight days. Survival was influenced by EPN species, growing media and days (GLM quasi-Poisson distribution:  $F^2 = 553355$ ,  $df = 840$ ,  $p < 0.01$ ) (Fig. 2; Table 3). Overall, there was a significant difference in the survival between the two EPNs species (GLM quasi-Poisson distribution:  $F^2 = 3242023$ ,  $df = 898$ ,  $p < 0.01$ ). The mean number of *S. kraussei* ( $9833 \pm 4.67$ ) was significantly higher than *H. bacteriophora* ( $9295 \pm 4.55$ ) (Tukey HSD test:  $p < 0.05$ ). The mean number of live EPNs reduced over time (Tukey HSD test:  $p > 0.05$ ), except for *S. kraussei* in COIR growing media where number remained similar (Tukey HSD test:  $p < 0.05$ ) (Fig. 2).

**Table 3.** The mean ( $\pm$  S.E.) of *Steinernema kraussei* and *Heterorhabditis bacteriophora* recovered from six different growing media 1 day, 7 days, 14 days, 21 days and 28 days post application. Different letters indicate significant differences between treatments (Tukey HSD test:  $p < 0.05$ ).

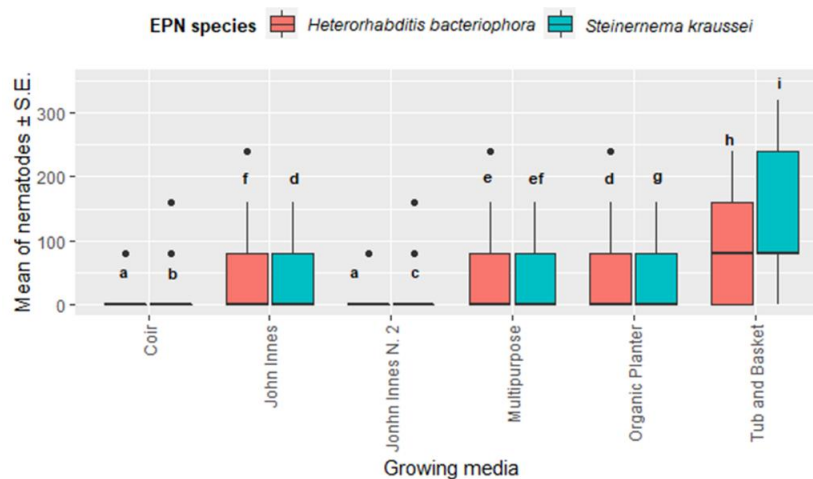
Growing Media	EPN species	Day 1	Day 7	Day 14	Day 21	Day 28
COIR	<i>S. kraussei</i>	12920 $\pm$ 756 <sup>ab</sup>	10860 $\pm$ 693 <sup>b</sup>	11800 $\pm$ 723 <sup>b</sup>	11140 $\pm$ 702 <sup>b</sup>	10700 $\pm$ 688 <sup>bd</sup>
	<i>H. bacteriophora</i>	18980 $\pm$ 916 <sup>a</sup>	18600 $\pm$ 907 <sup>a</sup>	7180 $\pm$ 564 <sup>cd</sup>	4320 $\pm$ 437 <sup>e</sup>	2120 $\pm$ 306 <sup>e</sup>
John Innes	<i>S. kraussei</i>	13380 $\pm$ 769 <sup>ab</sup>	14600 $\pm$ 804 <sup>a</sup>	10360 $\pm$ 677 <sup>bc</sup>	10300 $\pm$ 675 <sup>cd</sup>	8760 $\pm$ 623 <sup>de</sup>
	<i>H. bacteriophora</i>	17280 $\pm$ 874 <sup>a</sup>	8420 $\pm$ 610 <sup>ab</sup>	9220 $\pm$ 639 <sup>ce</sup>	4140 $\pm$ 428 <sup>f</sup>	3000 $\pm$ 364 <sup>f</sup>
John Innes N. 2	<i>S. kraussei</i>	12160 $\pm$ 733 <sup>a</sup>	14900 $\pm$ 812 <sup>a</sup>	5200 $\pm$ 480 <sup>b</sup>	1940 $\pm$ 293 <sup>c</sup>	1040 $\pm$ 214 <sup>c</sup>
	<i>H. bacteriophora</i>	15920 $\pm$ 839 <sup>a</sup>	14100 $\pm$ 790 <sup>b</sup>	6940 $\pm$ 554 <sup>b</sup>	1580 $\pm$ 264 <sup>c</sup>	600 $\pm$ 163 <sup>c</sup>
Multipurpose	<i>S. kraussei</i>	13320 $\pm$ 768 <sup>b</sup>	12600 $\pm$ 747 <sup>bc</sup>	9040 $\pm$ 632 <sup>cd</sup>	6800 $\pm$ 548 <sup>de</sup>	7240 $\pm$ 566 <sup>d</sup>
	<i>H. bacteriophora</i>	18320 $\pm$ 900 <sup>a</sup>	14900 $\pm$ 812 <sup>ab</sup>	12620 $\pm$ 747 <sup>bc</sup>	4060 $\pm$ 424 <sup>e</sup>	3180 $\pm$ 375 <sup>e</sup>
Organic Planter	<i>S. kraussei</i>	16727 $\pm$ 860 <sup>a</sup>	14300 $\pm$ 795 <sup>a</sup>	6540 $\pm$ 538 <sup>bc</sup>	5420 $\pm$ 490 <sup>bce</sup>	4200 $\pm$ 431 <sup>ce</sup>
	<i>H. bacteriophora</i>	20720 $\pm$ 957 <sup>a</sup>	13200 $\pm$ 764 <sup>a</sup>	8420 $\pm$ 610 <sup>b</sup>	4020 $\pm$ 422 <sup>de</sup>	3620 $\pm$ 400 <sup>de</sup>
Tub and Basket	<i>S. kraussei</i>	13740 $\pm$ 780 <sup>ab</sup>	14300 $\pm$ 795 <sup>bc</sup>	9260 $\pm$ 640 <sup>cf</sup>	7920 $\pm$ 592 <sup>def</sup>	5360 $\pm$ 487 <sup>df</sup>
	<i>H. bacteriophora</i>	18200 $\pm$ 897 <sup>a</sup>	13980 $\pm$ 786 <sup>ab</sup>	6920 $\pm$ 553 <sup>def</sup>	5280 $\pm$ 483 <sup>de</sup>	4780 $\pm$ 460 <sup>d</sup>



**Figure 2.** The mean ( $\pm$  S.E.) of entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* and *Steinernema kraussei*, recovered from experimental units containing six different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N. 2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental units ( $n = 5$ ) nematodes were counted 1 day, 7 days, 14 days, 21 days and 28 days post-application in the Survival Bioassay. Different letters indicate significant differences between treatments (Tukey HSD test:  $p < 0.05$ ).

#### 4.1.2 Dispersal Bioassay

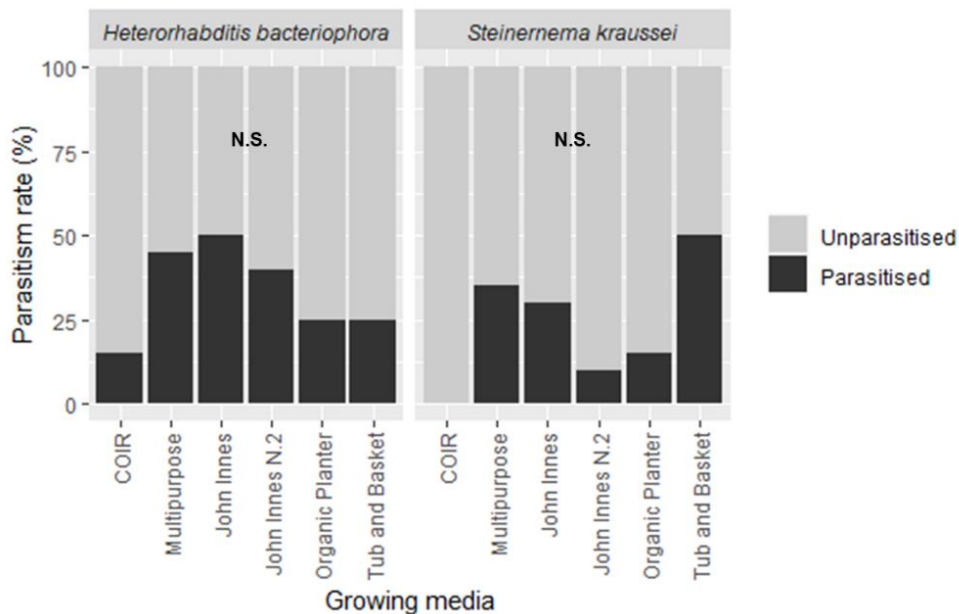
EPN dispersal was evaluated in a dispersal bioassay consisting of counting the number of *H. bacteriophora* and *S. kraussei* in the last segment of the experimental units with different growing media. Dispersal was significantly affected by growing media and EPN species (GLM quasi-Poisson distribution:  $F^2 = 50603$ ,  $df = 708$ ,  $p < 0.01$ ) (Fig. 3). The mean number of *S. kraussei* ( $125.33 \pm 1.44$ ) and *H. bacteriophora* ( $100 \pm 1.29$ ) were significantly higher in Tub and Basket compared all the other growing media used (Tukey HSD test:  $p < 0.05$ ). The lowest mean number ( $12 \pm 0.45$ ) of *S. kraussei* was in John Innes N. 2 (Tukey HSD test:  $p < 0.05$ ). The lowest mean number for *H. bacteriophora* was recorded also in John Innes N. 2 ( $6.67 \pm 0.33$ ) (Tukey HSD test:  $p < 0.05$ ) but this was not significantly different from the mean number of nematodes recorded in Coir (Tukey HSD test:  $p > 0.05$ ). Significant differences in the mean numbers of nematodes were found between *S. kraussei* and *H. bacteriophora* for all the peat-free growing media (Tukey HSD test:  $p < 0.05$ ), except for the Multipurpose (Tukey HSD test:  $p > 0.05$ ). The presence of a vine weevil larva in the experimental units significantly influenced the mean number of the nematode *S. kraussei* (GLM quasi-Poisson distribution:  $z = 6.53$ ,  $df = 1436$ ,  $p < 0.001$ ) ( $44.7 \pm 3.72$ ) compared to the control group (without larva) ( $15.1 \pm 2.17$ ). No significant differences were observed in the mean number of *H. bacteriophora* recovered from experimental units with or without a larva being present (GLM quasi-Poisson distribution:  $z = 1.63$ ,  $df = 1436$ ,  $p = 0.36$ ).



**Figure 3.** The mean ( $\pm$  S.E.) of entomopathogenic nematodes (EPNs), *Heterorhabditis bacteriophora* and *Steinernema kraussei*, counted in the bottom segment of experimental units when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N. 2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit (four segments of PEX pipe) 100  $\mu$ l of the nematode solutions (500 IJs) was added to corresponding units ( $n = 20$ ). Different letters indicate significant differences between the treatments (GLM quasi-Poisson distribution: Tukey HSD test:  $p < 0.05$ ).

#### 4.1.2.1 Parasitism Bioassay

Exposing vine weevil larvae to different EPNs and growing media in the dispersal bioassay had no effect on parasitism rate (GLM Binomial distribution:  $F^2 = -252.04$ ,  $df = 228$   $p > 0.05$ ) (Fig. 4). Parasitism rate varied between 0% (in Coir) and 50% (Tub and Basket) for *S. kraussei* and between 15 % (in Coir) and 50 % (in John Innes) for *H. bacteriophora*.



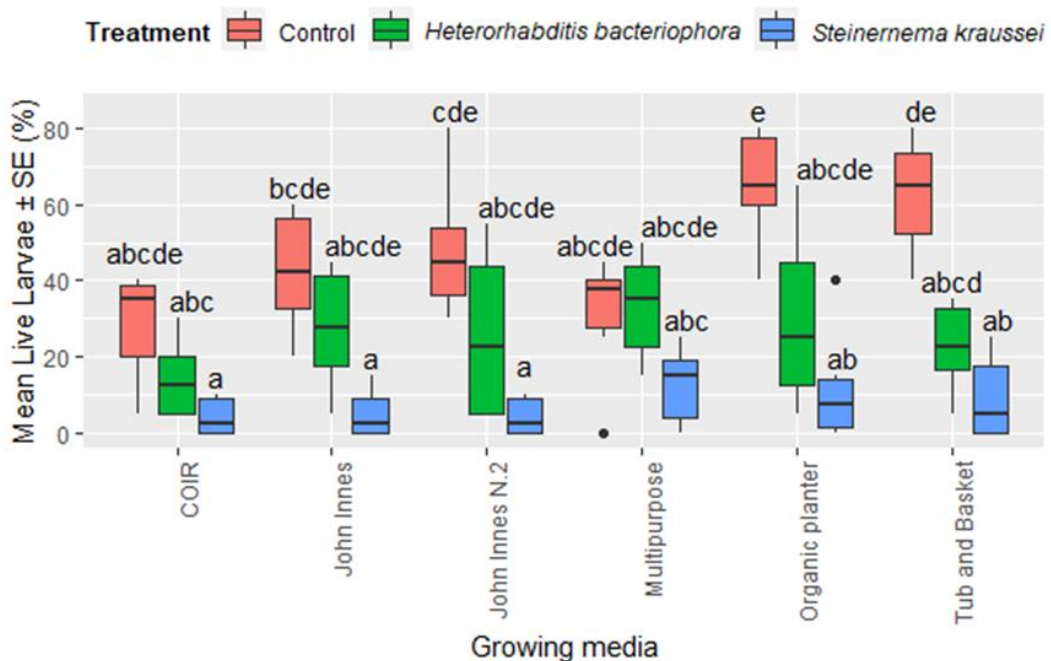
**Figure 4.** The parasitism rate (%) of vine weevil by *H. bacteriophora* and *S. kraussei* when testing in six different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit (four segments of PEX pipe) 100  $\mu$ l of the nematode solutions (500 IJs) was added to corresponding units ( $n = 20$ ). (GLM Binomial distribution:  $p < 0.05$ ). N.S. = not significant.

## 4.2 Glasshouse Experiments

### 4.2.1 Efficacy Bioassay

Exposing vine weevil to *S. kraussei* under glasshouse conditions significantly reduced the percentage of larvae recorded in Begonia pots (GLM with a quasi-Poisson distribution:  $t = -2.94$ ,  $df = 90$ ,  $p < 0.004$ ) compared to the control group (Fig. 5). In John Innes growing media the percentage of larvae in the control treatment ( $42.5 \pm 8.10$  %) was significantly higher than that in the treatment with *S. kraussei* ( $5 \pm 2.78$  %) (Tukey HSD test:  $p < 0.05$ ) (Fig. 5). In John Innes N. 2 the percentage of larvae in the control treatment ( $48.33 \pm 8.64$  %) was

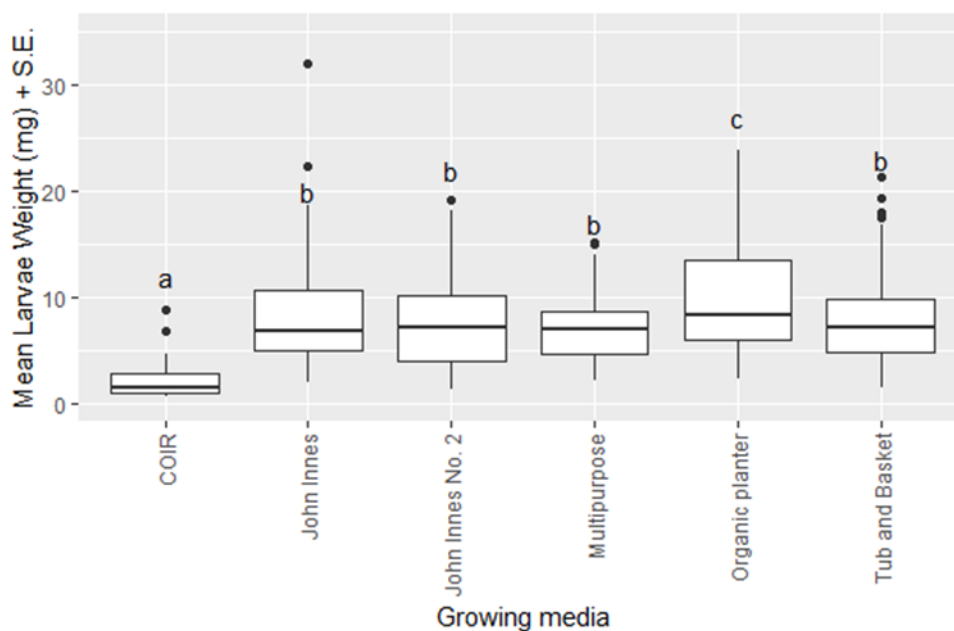
significantly higher than that in the treatment with *S. kraussei* ( $4.17 \pm 2.17$ ) (Tukey HSD test:  $p < 0.05$ ) (Fig. 5). The percentage of larvae in the control treatment ( $65 \pm 10.02$  %) was significantly higher than that in treatment with *S. kraussei* ( $11.67 \pm 4.24$ ) (Tukey HSD test:  $p < 0.05$ ) (Fig. 5) in the Organic Planter growing media treatment. In the Tub and Basket growing media the percentage of larvae ( $62.5 \pm 9.82$  %) in the control treatment was significantly higher than that in treatment with *S. kraussei* ( $9.17 \pm 4.10$  %) (Tukey HSD test:  $p < 0.05$ ) (Fig. 5). There were not significant differences in percentage of larvae recovered from Coir and Multipurpose between treatments (*S. kraussei* and control). The percentage of larvae recorded in Begonia pots treated with *H. bacteriophora* was not significantly different compared to that in the control group (GLM with a quasi-Poisson distribution:  $t = -1.72$ ,  $df = 90$ ,  $p = 0.09$ ).



**Figure 5.** Percentage (mean  $\pm$  S.E.) of vine weevil larvae recorded from experimental units (Begonia plant) treated with *S. kraussei*, *H. bacteriophora* or without EPN species (Control) when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). For each experimental unit 100 ml of the nematode solutions (25000 IJs) was added to corresponding units (number of replicates = 6). Different letters indicate significant differences between the treatments (GLM quasi-Poisson distribution: Tukey HSD test:  $p < 0.05$ ).

#### 4.2.2 Larval Weight

Exposing vine weevil to EPNs in an efficacy bioassay using different peat-free growing media influenced larval weight (Kruskal-Wallis:  $\chi^2 = 144.03$ ,  $df = 5$ ,  $p < 0.01$ ). Larvae recorded in Coir weighted significantly less ( $2.06 \pm 0.21$  mg) compared to the other growing media (Wilcoxon rank sum test:  $W < 0.001$ ) (Fig. 6). Whereas the larvae recorded in the Organic Planter growing media were significantly overweight ( $9.97 \pm 0.47$  mg) compared to the weight of the larvae recorded in the other growing media (Wilcoxon rank sum test:  $W < 0.001$ ). No significant differences in larval weight were recorded between the treatment with EPNs or without (control) (Kruskal-Wallis:  $\chi^2 = 0.04$ ,  $df = 1$ ,  $p = 0.83$ ).



**Figure 6.** Weight (mean  $\pm$  S.E.) of vine weevil larvae recorded from experimental units (Begonia plant) treated with *S. kraussei*, *H. bacteriophora* or without EPN species (Control) when testing different growing media: Cocogreen BerryFusion Coir (COIR), SylvaGrow with added John Innes (John Innes), SylvaGrow John Innes Number 2 (John Innes N.2), SylvaGrow Multipurpose (Multipurpose), SylvaGrow Organic Planter (Organic Planter) and SylvaGrow Tub and Basket (Tub and Basket). Different letters indicate significant differences between the treatments (Wilcoxon rank sum test  $W < 0.05$ ).

#### 5. Discussion

Entomopathogenic nematodes are an important management strategy for controlling insect pests in agriculture and horticulture (Lacey and Georgis, 2012). However, despite the imminent transition to peat-free growing media, little attention has been given to evaluating

the efficacy of established biocontrol techniques for managing belowground pests in alternative growing media.

In this study, higher survival of *S. kraussei* was recorded than for *H. bacteriophora* over time across different peat-free growing media. While both EPN species showed a rapid decline in numbers within the first seven days in all media tested, *S. kraussei* numbers remained relatively high in Coir growing media. This finding aligns with previous studies, which have shown that *Steinernema* species generally exhibit better survival compared than *Heterorhabditis* species (Abate *et al.*, 2019). For instance, Molyneux (1985) reported that *Steinernema* species had higher survival rates than *Heterorhabditis* species on sand. Similarly, Griffin (2015) and Smits (1996) reported an initial rapid decline in nematode numbers following application, followed by a more gradual decrease over the next two to six weeks post-application, consistent with the pattern observed in this study.

The efficacy of EPNs depends on a combination of soil or growing media conditions (soil/growing media type, pH, soil moisture) (Nielsen and Lewis, 2012), nematode foraging strategy, insect host species, host location, climate and application methods (Noosidum *et al.*, 2010). In particular, differences in EPN survival in different growing media is known to be influenced by different chemical and physical characteristics of growing media, including pH. It has been shown, for example, that the survival of *S. glaseri* and *S. carpocapsae* was similar at pH 4, 6 and 8 during the first four weeks, but *S. carpocapsae* survival was significantly greater than *S. glaseri* at pH 10 through sixteen weeks (Kung *et al.*, 1990). In another study Khathwayo *et al.* (2021) observed that Steinernematids survived better at a wider pH range from neutral to slightly alkaline pH in ammoniumacetate solutions compared with the Heterorhabditids. In this study, both EPNs survival in the most of growing media at a greater rate than that in Coir. This could be explained by pH levels, as some of the tested growing media, such as John Innes N. 2 and Tub and Basket, were among the most acidic. However, limited information is available regarding the impact of pH on the survival of entomopathogenic nematodes (EPNs) and their ability to infect hosts and reproduce after exposure. Thus, it would be valuable for future research to investigate the infectivity of surviving IJs following exposure to various pH levels.

Moisture has been recognised as one of the most important factors in the soil or growing media environment affecting EPN survival (Koppenhöfer and Fuzy, 2006; Nouh, 2022). Yadav and Lalramliana (2012) reported that the optimum soil moisture for *H. indica* was between 8 and 18 %, between 6 and 20 % for *S. thermophilum* and between 8 and 25 % for *S. glaseri*. Further, a minimum soil moisture level of 6% was found to be essential for achieving 100% host mortality across all three EPN species. Burman and Pye (1980) reported that high water content in soil may increase anaerobic regions, which induces inefficient EPN respiration and decreases survival. The observations in the present study might be explained

by this as Coir had the highest survival of EPNs and the lowest moisture content. However, further research is needed to understand better the role of soil or growing media moisture on EPN survival and optimise conditions for EPN efficacy.

Dispersal and host finding behaviour of EPNs have been extensively studied across various soil types, with several EPN species exhibiting differences in both dispersal patterns and host-finding strategies (Lewis *et al.*, 2006). In this study, the dispersal bioassay was designed to evaluate the ability of *S. kraussei* and *H. bacteriophora* to move vertically through the different peat alternative growing media. Results indicated that more IJs of both species were found in the bottom layer of the column filled with Tub and Basket. On the other hand, all nematode moved less in John Innes N. 2 and Coir. The lower ability of IJs to move through John Innes N. 2 could be due to bulk density, as John Innes N. 2 had the highest value and it has previously been reported to reduce the dispersal of EPNs (Portillo-Aguilar *et al.*, 1999). In addition, John Innes N. 2 consists of sterilised loam, a fine particle material, and smaller particle sizes are known to limit EPN movement. For instance, Portillo-Anguillar *et al.* (1999) found that EPN movement in loam was lower than in sandy loam. Similarly, Kung *et al.* (1990b) showed that porosity affects the survival and pathogenicity of two Steinernematid species, using substrates like sand, sandy loam, clay loam and clay. Substrates with lower porosity (clay loam and clay) significantly reduced EPN survival, likely due to poor aeration and restricted movement. Likewise, El-Borai *et al.* (2012) assessing the efficacy of *H. indica*, *S. diaprepesi* and *S. riobrave* against the citrus root weevil (*Diaprepes abbreviatus* L.; Coleoptera: Curculionidae) in different sandy growing media (coarse sand, fine sand, and sandy loam), found that EPNs were more effective in the medium with the largest particle size and greatest porosity (coarse sand). This is similar to the observed differences between John Innes N.2 and John Innes, as the inclusion of sand in John Innes increases loam porosity (Portillo-Aguilar *et al.*, 1999). Results from this study are, therefore, in agreement with those from other studies which have showed that EPN dispersal is positively correlated with the percentage of sand, silt and organic matter, and negatively correlated with the percentage of clay (Koppenhöfer and Fuzy, 2006; Noosidum *et al.*, 2010). This is likely because nematode movement is restricted in finer textured soils or growing media due to smaller pores which result in the reduction of nematode respiration, nematode survival and their efficacy (Koppenhöfer and Fuzy, 2007).

Ansari and Butt (2011) reported the maximum recovery of EPNs, including *Steinernema feltiae*, *S. carpocapsae*, *S. kraussei*, *Heterorhabditis megidis*, *H. bacteriophora* and *H. downesi*, after twenty-four hours in bioassay tubes filled with Coir rather than peat and bark. In our study, although more IJs were recovered from the survival bioassay with Coir, the number of nematodes counted in the dispersal bioassay tubes filled with Coir were lower. However, Ansari and Butt (2011) EPN dispersal in tubes measuring between two and four cm,

whereas in the present study, IJs were tested in experimental units measuring between six and eight cm. Another factor that may explain the differences in results between studies is the variation in moisture levels, as water film thickness is known to influence EPN movement (Wallace, 1958). Soils and growing media with higher porosity and suitable aqueous spaces, like Coir, have been shown to enhance the survival, mobility and effectiveness of EPNs compared to media like composted green waste, peat and bark (Ansari and Butt, 2011b). However, Ansari and Butt (2011) did not report the moisture content of the media used in their experiment, making it difficult to assess this factor. Therefore, future studies are needed to investigate the efficacy of EPNs in Coir growing media.

Presence of a host insect has been known to affect dispersal (Ansari and Butt, 2011). In this study, significant differences in the number of *S. kraussei* were recorded when experimental units in the Dispersal Bioassay were baited with vine weevil larva compared to the control. This is likely due to responses to host cues that have been suggested to be important in host recognition and finding. For example, carbon dioxide has been shown to be an important cue for EPNs in locating uninfected hosts (Ramos-Rodríguez *et al.*, 2007) as well as plant derived cues (van Tol *et al.*, 2001; Zhang *et al.*, 2021). Inter-specific differences in response to both contact and volatile cues have also been observed in EPNs. For example, Lewis *et al.* (1992) showed that the EPN *S. glaseri* was able to select chemical host cues from the Japanese beetle (*Popillia japonica* Newman; Coleoptera: Scarabaeidae) and the beet armyworm (*Spodoptera exigua* Hübner; Lepidoptera: Noctuidae) for host location more effectively than *S. carpocapsae*. In this study, although higher numbers of *S. kraussei* were recorded in the final segment of the column, little is known about the olfactory cues that attract this EPN species to vine weevil larva or the behavioural responses these cues elicit. Future investigations are needed to provide a better understanding of how EPNs locate hosts such as vine weevil and discriminate among potential hosts. This information would provide the basis to improve the efficacy of EPNs as biocontrol agents.

Although the efficacy of entomopathogenic nematodes has been well-studied under laboratory conditions, limited information exists regarding their dispersal and distribution patterns when applied inundatively for vine weevil control in the field. One of the major challenges in using EPNs as biological control agents is their poor post-application survival (Abate *et al.*, 2019). Previous studies have identified several factors that may influence EPN efficacy in field conditions, including biotic environmental factors such as plant habitat type (Bal *et al.*, 2017), abundance of host (Půža and Mráček, 2005), spatial distribution in the soil profile (Bal *et al.*, 2017), abiotic edaphic factors such as soil texture and moisture (Georgis and Poinar, 1983; Kung *et al.*, 1991) and the combinations of these factors (Hoy *et al.*, 2008). Environmental factors such as soil or growing media type, pH, relative humidity, moisture content, temperature and ultraviolet radiation (Abate *et al.*, 2019) have shown also to have a

significant impact on EPNs efficacy (Stuart *et al.*, 2015). Such environmental factors are thought to influence the amount of lipids (Andaló *et al.*, 2011), a major stored nematode energy reserve in their bodies, as well as the rate at which this energy reserves are depleted (Grewal, 2000). In our study, *S. kraussei* has shown overall to effectively control vine weevil under glasshouse conditions. In contrast, the percentage of larvae recorded in Begonia pots treated with *H. bacteriophora* was not significantly different from that in the control group. This result, however, may have been influenced by the low temperatures (minimum growing media temperature, overall, below 7 °C; Table 1) during the experiments, which were conducted in winter. Therefore, further studies are needed to understand performance, adaptability and potential use of *H. bacteriophora* against vine weevil under warmer field conditions.

In Coir, recovered larvae were fewer in number and smaller compared to those in other growing media. These findings are in contrast with previous studies where vine weevil larvae were reported to thrive in Coir growing media compared with peat and peat blends or bark (Shah *et al.*, 2007b). Therefore, further investigation is needed to understand the impact of peat alternative growing media, including Coir, on vine weevil survival and development.

While peat-free growing media offer environmental benefits and align with sustainable practices, their impact on the efficacy of EPNs needs thorough investigation. This study provides evidence to suggest that peat-free growing media may impact the survival and dispersal of EPNs. Understanding how chemical and physical properties of growing media such as pH, moisture, bulk density, porosity *etc.* affect EPNs performance is crucial for optimizing pest management strategies in a peat-free context.

# Chapter 7: General Discussion

## 1. Current Knowledge and Future Perspectives

### 1.1 The Importance of Visual Characteristic of Vine Weevil Monitoring Tool Efficacy

Currently available vine weevil monitoring tools lack the required sensitivity to reliably indicate pest presence or density under field conditions (Roberts *et al.*, 2019a). Development of improved monitoring systems is currently hindered by a lack of knowledge of whether vine weevil adults select a monitoring tool based on its visual appearance. This thesis provides a first step toward understanding the visual preferences of adult vine weevil as well as evidence of the importance of visual ecology in the development of effective and reliable vine weevil monitoring tools. In Chapter 2, results showed that monitoring tool colour, height and diameter all influenced monitoring tool efficacy, with individuals exhibiting a preference for dark, tall and wide monitoring tools. Furthermore, monitoring tool density per unit area affected the proportion of vine weevil population recorded by monitoring tools. By contrast, within the tested conditions, crop density was found to have no effect on monitoring tool efficacy.

Chapter 2 identified the influence of multiple factors (e.g., colour, height, diameter), therefore, it would be valuable to investigate how these variables interact to determine whether certain combinations increase or decrease monitoring tool efficacy, helping to identify an optimal tool design (Reddy *et al.*, 2011). To do this, it would be useful to assess interactions and their effects on vine weevil attraction by carrying out experiments to test combinations of these variables (e.g., dark + tall + wide vs. light + short + narrow). The preference for dark colours, tall, wide monitoring tools suggests that vine weevil visual preferences play a key role. However, future studies should determine whether certain patterns/colours mimic natural habitats (Silva *et al.*, 2018) or are inherently attractive, enhancing monitoring tool design. In doing this, it might be useful to test a broader range of colours (e.g., shades of green, blue and black) and patterns (e.g., stripes, spots) to identify if specific visual cues are more attractive.

Although crop density was not found to affect monitoring tool efficacy under the tested conditions, it would be important to assess if placement within high-density crops (e.g., along rows, near plant bases) (Spear-O'mara and Allen, 2007) might improve detection under specific agricultural conditions. It is recommended to test monitoring tool efficacy across diverse crop densities (low, medium, high) in different settings (e.g., open fields, greenhouses) and seasons to ensure robust conclusions. Similarly, it would be important to complete additional tests to determine the best monitoring tool density per unit area for capturing the

maximum proportion of vine weevil adults. For this, it would be useful to measure the proportion of vine weevil adults captured in monitoring tools at varying densities (e.g., low, medium, high densities) in the same field or greenhouse. It is recommended also to investigate whether the spatial arrangement of traps (e.g., evenly spaced, clustered, random) affects monitoring tool efficacy as well as to assess whether optimal trap density (Vidyasagar *et al.*, 2016) differs depending on crop type or planting density by testing, for example, different monitoring tools densities in fields or greenhouses with varying crop types (e.g., strawberries, ornamentals, or other vine weevil host plants).

To use monitoring tools under field conditions careful consideration should be also given to the duration of the monitoring period. It is necessary, therefore, to evaluate how the efficacy of monitoring tools changes over time (e.g., after weeks/months of exposure to environmental factors like UV light, rain, or soil contact).

While Chapter 2 focuses on visual preferences, combining visual and olfactory cues could significantly enhance monitoring tools. It is recommended to further explore how vine weevil visual preferences are influenced by olfactory cues and identify potential synergistic effects (Pope and Roberts, 2022). Further studies are needed to test monitoring tools with dark colours or preferred dimensions in combination with olfactory attractants (e.g., pheromones and volatiles from host plants).

By addressing these research areas, it will be possible to develop more effective monitoring and control strategies for vine weevil, ultimately reducing their impact on crops and ornamental plants.

## **1.2 Potential of Semiochemicals from Host Plants and Conspecifics That May Be Used to Improve Monitoring Tool Efficacy**

One aspect of vine weevil biology where improved knowledge would directly benefit integrated pest management programmes is the chemical ecology of this species. Key to developing improved monitoring tools is the availability of a semiochemical lure (Hallett *et al.*, 1999; Cross *et al.*, 2006; St Onge *et al.*, 2018). To date, knowledge of vine weevil responses to semiochemicals has focused on plant volatiles, in particular two compounds identified from the spindle tree (*Euonymus fortunei*), (*Z*)-2-pentenol and methyl eugenol (van Tol *et al.*, 2012; 2020). Roberts *et al.* (2019a) showed that vine weevil adults preferentially used monitoring tools baited with the spindle tree or yew (*Taxus baccata*) foliage compared to unbaited monitoring tool. This preference was confirmed in Chapter 3 where monitoring tools baited with yew were preferred by vine weevil adults compared to unbaited monitoring tools under glasshouse conditions. However, although these studies suggest that vine weevil adults use

olfactory cues to locate host plants, further studies would be needed to understand which plant volatiles elicit vine weevil responses and the correct ratio of these components when using blends of plant volatiles.

Despite the limited success to date in the identification and commercial adoption of vine weevil lures based on plant VOCs (volatile organic compounds) there remains great potential should an aggregation pheromone be identified due to the likely stronger behavioural response that this would elicit (Abbas *et al.*, 2006; Pajares *et al.*, 2010). Here, several compounds have been identified that are believed to be produced by vine weevil adults (Pickett *et al.*, 1996) but behavioural responses to these compounds remain to be determined. Previous studies (van Tol *et al.*, 2004; Nakamuta *et al.*, 2005) reported positive behavioural responses between conspecifics, indeed Pickett *et al.* (1996) suggested that previously occupied refuges stimulate aggregation behaviour. In Chapter 3, it was shown that the presence of weevils in monitoring tools alone did not significantly affect the response of conspecifics. However, when monitoring tools were baited with both yew and conspecifics, higher numbers of vine weevil adults were recorded in these monitoring tools than in monitoring tools left unbaited or containing only yew. This suggests that vine weevil adults may use, as olfactory cues, volatiles associated with frass or induced by conspecifics during feeding. In fact, Pope and Roberts (2022) suggested that aggregation and conspecific attraction may be mediated by frass or other nonchemical cues.

It would be important to determine whether frass itself or volatiles induced by conspecific feeding are responsible for the observed aggregation behaviour. Identify specific volatile compounds that mediate conspecific attraction could be used to enhance the efficacy of monitoring or trapping tools (van Tol *et al.*, 2004; Fezza *et al.*, 2023). For this reason, tests might be carried out to test frass from vine weevils feeding on different host plants to determine whether host-specific differences influence attraction. It would be necessary to identify also these volatile compounds derived from frass using, for example, gas chromatography-mass spectrometry (GC-MS). Similarly, further investigations are needed to determine if combining host plant and frass volatiles creates a synergistic effect (Addesso *et al.*, 2011) that could improve the efficacy of monitoring tools. Experiments could be carried out to test monitoring tools baited with host plant volatiles (e.g., yew) alone, frass alone and a combination of both. After that, it would be useful to assess whether the combination of cues enhances attraction compared to either cue alone.

Further studies are needed to understand whether nonchemical cues also play a role in aggregation and how they interact with olfactory signals (van Tol *et al.*, 2004). This might be done, by comparing weevil responses to monitoring tools containing only visual/tactile cues (e.g., frass residue or weevil silhouettes) vs. those containing frass or live conspecifics as well as testing whether weevils prefer monitoring tools that mimic the physical appearance of

conspicuous or frass deposits. These follow-up studies will provide an improved understanding of the role of conspecifics and frass in vine weevil behaviour, helping to optimise monitoring tools and potentially develop novel vine weevil control strategies.

### **1.3 Investigate the Potential of Semiochemicals from Apple-Based Products That May Be Used to Improve Monitoring Tool Efficacy**

Volatile organic compounds (VOCs) emitted by fruits play a crucial role in attracting insect pests. Apples, like many other fruits, emit a complex blend of volatiles that can be used to develop effective lures for monitoring and controlling pest populations, including vine weevil. Chapter 4 shows that vine weevil adults show positive behavioural responses to apple sauce when laboratory and glasshouse experiments were carried out. However, further studies are needed to identify the specific volatile organic compounds (VOCs) in apple sauce that attract vine weevils. It is recommended to collect headspace volatiles from apple sauce (e.g., solid-phase microextraction (SPME)), analyse the collected volatiles using gas chromatography-mass spectrometry (GC-MS) and conduct behavioural assays (e.g., Y-tube olfactometer) to test the response of vine weevils to individual VOCs and blends. It would be useful to determine the optimal concentration of identified apple VOCs that elicit the strongest response in vine weevil adults under laboratory and field conditions. Further investigations would need to investigate whether combining apple VOCs with other known attractants (e.g., plant-derived VOCs) enhances vine weevil behavioural responses, as showed in these preliminary studies.

Synthetic blends can be designed to remain effective for weeks or months, unlike natural lures that degrade quickly (Rosa *et al.*, 2017). Therefore, it would be useful to create synthetic blends of apple sauce VOCs as well as to determine the optimal ratio of the identified VOCs to maximise vine weevil attraction. This can be done by testing these blends in behavioural assays (laboratory and glasshouse experiments).

By leveraging the responses of this weevil to apple volatiles, growers may be able to effectively monitor and control pest populations, for example through the use of auto-dissemination biopesticides (Pope *et al.*, 2018). Continued research will further enhance the efficacy and adoption of apple volatile-based trapping systems in vine weevil management programme.

## 1.4 Efficacy of a Commercially Available Garlic-Based Bioinsecticide Against Vine Weevil Eggs and Larvae

Botanical bioinsecticides offer a promising and sustainable approach to managing insect pests (Lengai *et al.*, 2020). In particular, garlic-based oils and extract formulations have been reported to possess insecticidal and repellent properties for a number of economically important insect pests, including weevils (Weathersbee and Tang, 2002; Parwada *et al.*, 2018). In Chapter 5, studies were carried out to evaluate the efficacy of a garlic-based product commercially available in the UK, Pitcher GR<sup>®</sup>, as a sustainable management option for vine weevil control. This garlic-based product showed to be effective against vine weevil eggs and larvae under laboratory conditions, reducing egg hatch and larval survival. However, its efficacy under glasshouse conditions decreased significantly, suggesting that its use requires careful consideration of product properties, application methods and integration into broader pest management strategies. Little is known about the movement of vine weevil larvae and how this might impact the efficacy of this bioinsecticide. Further investigations are, therefore, needed to understand how larval movement within the soil affects their exposure to Pitcher GR<sup>®</sup> and its efficacy. It is recommended to carry out experiments using soil observation chambers (Furmanczyk *et al.*, 2021) with transparent sides to track the movement of larvae in treated versus untreated soil as well as to carry out experiments with varying soil types, moisture levels and organic matter to assess how these factors influence larval behaviour and the bioinsecticide's performance. These studies would help to identify factors that reduce larval exposure to the garlic product and optimise its application for greater efficacy.

There is currently no information about the effect of the Pitcher GR<sup>®</sup> product on vine weevil adults, and, therefore, there is a need to determine what effects this product has on adult survival, feeding and egg-laying. It would be important to complete experiments to: (i) evaluate the impact of Pitcher GR<sup>®</sup> on adult survival rates when exposed to treated plant surfaces or soil, (ii) assess feeding deterrence by providing treated and untreated foliage to adults and measuring feeding damage over time, (iii) investigate its effects on egg-laying behaviour and egg production by treating plants and soil with Pitcher GR<sup>®</sup> and recording the number of eggs laid by adults. The results of these studies will provide important insights into the product's potential to reduce adult vine weevil activity and reproduction.

Continued research and development on garlic-based bioinsecticides, along with practical field application techniques, will be essential for optimising effective vine weevil control. In addition, further investigations are needed to evaluate the use of garlic-based bioinsecticides in conjunction with other IPM strategies such as the use of entomopathogenic nematodes (EPNs). Preliminary investigations in Chapter 5 showed that Pitcher GR<sup>®</sup> reduced the survival of the EPN, *Steinernema kraussei*, under laboratory conditions, suggesting a low

compatibility between EPN use and the garlic-based bioinsecticide. However, further research is needed to fully investigate the compatibility between garlic-based product and EPNs within vine weevil IPM programme.

### **1.5 Efficacy of Entomopathogenic Nematodes Against Vine Weevil Larvae When Applied to Peat-Free Growing Media**

In a search of sustainable alternatives to the use of synthetic chemical insecticides, EPNs have emerged as promising biological control agents against major insect pests, including weevils. Almost thirty families of EPNs are reported to be pathogenic to many insect species (Kapranas *et al.* 2017; Dlamini *et al.* 2019). For vine weevil, a large number of studies have reported that EPNs are effective under laboratory as well as field conditions (Lola-Luz and Downes 2007; Haukeland and Lola-Luz 2010). However, it is known that the efficacy of EPNs in controlling insect pests is influenced by a complex interplay of environmental conditions, soil and growing media properties, host factors, nematode species and strains, application methods and interactions with other pest control strategies (Belien, 2019; Devi, 2024). In Chapter 6, studies focused on evaluating the efficacy of EPNs on six commercially available peat alternative growing media. Results showed that EPNs were able to survive in all the growing media types tested. However, dispersal was affected by growing media, with Coir and John Innes Number 2 having a negative effect on EPNs. An improved understanding of how the physio-chemical properties of peat-free growing media influences EPN survival, dispersal and virulence, would enhance the performance of these controls in sustainable pest management programmes. It is suggested to carry out further studies to analyse the physical and chemical properties of these growing media (e.g., particle size, porosity, pH, water-holding capacity, organic matter content) and how they impact EPN survival, movement and virulence (Kaya, 1990; Salame and Glazer, 2015; Khumalo *et al.*, 2021).

Under glasshouse conditions, application of *S. kraussei* gave significantly greater efficacy in managing vine weevil across all tested growing media. By contrast, the percentage of larvae recorded in Begonia pots treated with *H. bacteriophora* was not significantly different compared to that in the control group. However, this outcome may have been influenced by the low temperatures during the experiments, which were conducted in winter. It is well established that the optimal temperature range for *H. bacteriophora* is between 16 and 35 °C, potentially limiting its performance under the experimental conditions (minimum growing media temperature, overall, was below 7 °C). To fully evaluate the efficacy of this EPN species, future studies should focus on field trials carried out across its tolerated temperature

range and seasonal conditions, as well as in different growing media, to better understand their performance and adaptability under field scenarios.

## **2. Developing Improved Tools with Which to Reduce Vine Weevil Damage**

While the last 30 years has undoubtedly seen improved knowledge of vine weevil biology, several key areas still need more research. These gaps include creating a detailed annotated genome, understanding how the pest interacts with various crops and control methods at a molecular level, developing summary models for vine weevil development and enhancing our knowledge of the pest's chemical ecology to inform future forecasting, monitoring, prevention and control strategies.

One aspect of vine weevil biology has been largely neglected with comparatively few studies investigating the molecular biology of this species. This means that despite the economic importance of this species there is still no annotated genome available and, therefore, it is largely unknown how vine weevil interacts at a molecular level with the crop environment or controls used against it. For other generalist arthropods, such as the two-spotted spider mite (*Tetranychus urticae* Koch; Trombidiformes: Tetranychidae), publication of the completely sequenced and annotated genome has revealed strong signatures of polyphagy and detoxification genes linked to feeding on different host plants (Grbić *et al.*, 2011). Furthermore, transcriptomic analysis of mites feeding on different hosts shows how these arthropods respond to changes in their environment. Importantly, with a published genome available the detoxification genes linked to feeding on different host plants have been identified. In order to manipulate mite gene function or protein activity, Suzuki *et al.* (2017) described protocols to deliver small molecules into the mite. This represents a first step to develop a reverse genetics platform to modulate gene expression or protein activity as well as a high throughput screening for mite control. Similarly, the publication of the first genome assembly of the red palm weevil (*Rhynchophorus ferrugineus* Olivier; Coleoptera: Curculionidae) (Hazzouri *et al.*, 2020), one of the most invasive insect pests affecting date palms globally (El-Shafie and Faleiro, 2020), provides a crucial foundation for genetic modifications or gene silencing approaches. For example, Rasool *et al.* (2020) showed that silencing the reproduction control gene *vitellogenin* (*Vg*), a major yolk protein precursor critical for oogenesis, impacts red palm weevil female performance. Most efforts have been focused also on the use of genetic markers to study red palm weevil expansion or population structure by the use of RAPD (random amplified polymorphic DNA) markers (Gadelhak and Enan, 2005; Al-Ayied *et al.*, 2006; Wang *et al.*, 2013). Hence, this genomic resource enhances our ability to develop targeted strategies for controlling pests, potentially mitigating their devastating impact on economically important crops.

Another area in which further work is needed is vine weevil thermal biology. While the last 30 years has undoubtedly seen improved in this area (Son and Lewis, 2005a, b) (Chapter 1) future studies should focus on developing forecasting tools with which to determine key developmental stages in vine weevil life cycle as well as population growth within crop habitats. In particular, it would be useful to develop a summary model for complete development from egg to reproductive adult (Pope and Roberts, 2022).

A second aspect of vine weevil biology where improved knowledge would directly benefit pest management programmes, is the chemical and visual ecology of this species. By exploring the chemical cues that vine weevils use for host plant and conspecific location, it would be possible to develop more targeted and effective monitoring and control strategies. Improved knowledge in these areas would provide valuable tools for integrated pest management programmes.

The switch from the use of persistent synthetic insecticides for the control of vine weevil to the reliance on the use of invertebrate biological control agents such as *S. kraussei* or *H. bacteriophora* is a major step toward development of a sustainable crop protection system. Despite this, there are limitations in the use of currently available microbial biopesticides, such as entomopathogenic fungi (EPFs), when used in the climatic conditions typically found in temperate regions such as the UK. In order to address this problem, it has been suggested that selection of locally adapted isolates may be an important step to consider (Klingen *et al.*, 2015) in future studies.

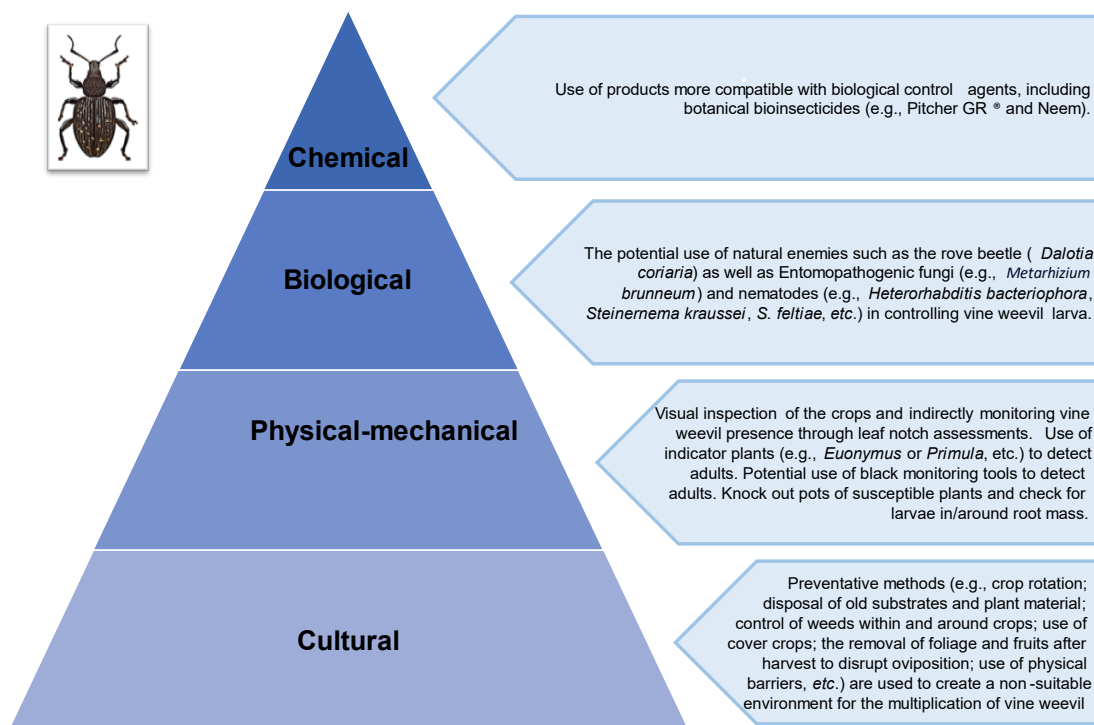
Another area in which further development of existing controls is required is to complement existing EPN and garlic-based controls that target vine weevil eggs and larvae with environmentally sustainable controls that target adults. Similarly, despite the potential of an autodissemination system for entomopathogenic fungi has previously been demonstrated (Pope *et al.*, 2018) further work would be required to develop this into a usable tool within an IPM programme. Furthermore, an autodissemination or mass trapping system are only likely to be truly effective when a highly effective lure can be developed.

Research in all the areas previously mentioned is crucial for reducing the economic impact of vine weevils by implementing effective and integrated approaches. To successfully integrate IPM tools, including automatic monitoring, forecasting and dynamic economic thresholds, more focus on data science is needed (Pope and Roberts, 2022). For instance, using sensors in crops would provide real-time environmental data to predict pest population growth, guiding when and how to apply control measures. Additionally, developing machine learning models to identify vine weevil adults entering monitoring devices (Roberts *et al.*, 2022) would allow growers to remotely track pest populations and receive timely alerts for control interventions.

### 3. Integrated Available Management Tools into IPM Programmes

There is a current lack of IPM systems research and instead a focus on single technologies to achieve effective control of a pest even when the controls used may be considered IPM compatible. The current approach to vine weevil control, therefore, does not consider or try to benefit from the synergistic interactions that may result from selectively and intelligently combining these tools (Thomas and Waage, 1996; Stenberg, 2017). A decision framework for the management of vine weevil has been developed based on the tools available and adapted for crop type (Bennison *et al.*, 2014). Similarly, Pope and Roberts (2022) proposed an IPM programme for vine weevil in strawberry, involving identifying and monitoring, thresholds, forecasting, prevention, control and evaluation.

These sequential management decisions may then be seen as providing a framework on which to base and further develop IPM programmes and importantly to recognise where gaps remain and how development of new tools may be incorporated (Fig. 1, Table 1).



**Figure 1.** Diagram showing IPM strategies adopted for vine weevil (*Otiorhynchus sulcatus*) control.

**Table 1.** Comparative table between IPM strategies available for vine weevil control

	<b>30 years ago (Moorhouse <i>et al.</i> 1992)</b>	<b>Present</b>	<b>Future prospective/ Gaps</b>
Biology	Many aspects of vine weevil biology understood	Improved understand of thermal biology but comparatively little the understanding of chemical ecology and molecular biology	Improved understanding of vine weevil chemical and visual ecology as well as molecular biology of this species required
Monitoring- adults	Not mentioned, only the use of physical barriers and traps were referred to as physical controls and considered unattractive and ineffective	Night-time assessments completed using a torch or by shaking/ tapping plants. Look for the characteristic leaf notches along leaf margin. Use of indicator plants to record characteristic feeding damage used in some situations	Development of effective monitoring tool required in which a vine weevil specific lure is used
Monitoring- larvae	Not mentioned	Knocking out pots of susceptible plants and checking for larvae in/around root mass	Acoustic monitoring techniques to be made commercially available
Thresholds	Not mentioned only number of larvae likely to cause damage to crops mentioned	Thresholds are currently based on numbers of larvae per plant used rather than the number of adults	Development of dynamic economic thresholds required and these should be appropriate for use with biological as well as chemical plant protection products
Forecasting	Not mentioned	Progress has been made in developing population models for vine weevil, but these have not been made commercially available	Potential forecasting systems based on wireless in-crop sensors could be developed
Preventive control	A range of preventative controls mentioned, including: <ul style="list-style-type: none"> <li>- Use of crop rotation</li> <li>- disposal of old substrates and plant material</li> <li>- control of weeds within and around crops</li> <li>- use of cover crops such as white clover</li> <li>- the removal of foliage and fruits after harvest to disrupt oviposition</li> <li>- use of physical barriers</li> <li>- potential of plant resistance</li> </ul>	Lack of progress in developing preventative controls over last 30 years	Develop varieties of soft fruit and ornamental crops with resistance to vine weevil, utilising known resistance traits or through molecular approaches to plant breeding, e.g. CRISPR
Chemical control- adults	Foliar application of synthetic chemical insecticides (e.g., carbofuran, dieldrin, pyrethroid, diflubenzuron, etc.)	No synthetic insecticides approved for the control of vine weevil adults in the UK	Development of selective synthetic chemical insecticides that are compatible with biological controls that may also be used within crops

Chemical control-larvae	Application of larvicides (e.g., organochlorine insecticides etc.) by soil incorporation, drench application, dust or microencapsulation	No synthetic insecticides approved for the control of vine weevil larvae in the UK. However, Pitcher GR® (MAPP 18126; EAMU 2018-3744) could be used for early instar larvae in containerised ornamental plants	Development of selective synthetic chemical insecticides that are compatible with biological controls that may also be used within crops
Natural enemies	Highlighted the role of insectivorous mammals, reptiles and amphibians, birds and invertebrates all mentioned as part of vine weevil biology but not as potential controls	No commercially available natural enemies used against vine weevil. However, preliminary studies have demonstrated the potential of the rove beetle ( <i>Dalotia (Atheta) coriaria</i> ) in controlling vine weevil larvae	Further studies on the management of crop environments to increase numbers of natural enemies as well as the potential use inundative releases of invertebrate natural enemies for the control of this pest
Entomopathogenic nematodes	Heterorhabditid and Steinernematid nematodes already commercially available and used to control larvae, but little known about the impact of environmental variables on nematodes efficacy.	Improved awareness on the impact of environmental variables on nematode efficacy with the exception of peat free substrates. Nematode applications recommended in spring and autumn when conditions permit. Development of a 'little and often approach' for the application of nematodes between April and October	Further work to investigate how the selection of locally adapted isolates may increase the efficacy and persistence of nematodes
Entomopathogenic fungi	Only brief mentioned of the use of some species (e.g., <i>M. anisopliae</i> and <i>B. bassiana</i> ) against vine weevil larvae. No information on how environmental variables may influence the efficacy of these species of fungi	Entomopathogenic fungi widely used to control vine weevil larvae through incorporation of product into growing media for plants potted in the spring/summer. Minimum temperature required for activity against larvae is 15°C and understand that these products are unlikely to be effective in the autumn against larvae hatching from September to November in many regions	Potential of an autodissemination system for the use of entomopathogenic fungi against vine weevil adults requires further investigation. Further work to investigate how the selection of locally adapted isolates may increase the efficacy and persistence of entomopathogenic fungi. Mycorrhizal fungi have been shown to provide control of vine weevil larvae feeding on the roots of plants but a lack of research in this area
Endosymbiotic bacteria	Only briefly mentioned	<i>Wolbachia</i> infections have been reported in vine weevil and are thought to influence vine weevil egg viability.	Additional research is needed to understand the roles of endosymbiotic bacteria in vine weevil biology and how this may be exploited within IPM programmes

#### 4. Conclusion

The continued importance of vine weevil as a pest of economically important crops is evidenced by the large number of studies (> 200) investigating the biology and control of this

pest over the last 30 years. This research has directly led to the commercial adoption of environmentally-sound controls, most notably the use EPNs such as *S. kraussei* and *H. bacteriophora* to control vine weevil larvae. Despite this, if we consider IPM as “a holistic ‘approach’ or ‘strategy’ to combat plant pests and diseases using all available methods, while minimizing applications of chemical pesticides” (Stenberg, 2017) then it is clear that vine weevil control still falls short of true IPM. This is largely due to the absence of preventative methods, effective monitoring of vine weevil populations as well as known natural enemies, the use of economic thresholds, and the simultaneous management and integration of tactics (Kogan, 1998) in existing management programmes.

Despite the fact that current control of vine weevil populations falls short of being considered an example of true IPM, the fact that ‘standard practice’ in many commercial crop situations is to now the use EPNs such as *S. kraussei* or *H. bacteriophora* to control vine weevil larvae gives encouragement that more comprehensive IPM programmes could be developed. However, for this to happen the use of EPNs would have to be seen as one component of an integrated programme of crop protection measures rather than as a direct replacement for withdrawn conventional synthetic insecticides. At the same time, use of reliable pest monitoring, pest forecasting, economic thresholds and a range of cultural controls should complement and, in some cases, determine the use of these environmentally-sound controls.

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